

MOLECULAR CLONING OF BRUCELLA ABORTUS
GENES IN A COSMID VECTOR AND THEIR
EXPRESSION IN ESCHERICHIA COLI

By

MARY SHOKRALLA SEDRA
B
Bachelor of Medicine and Surgery
University of Alexandria
Alexandria, Egypt

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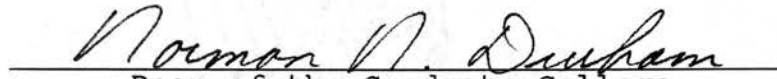
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Thesis Approved:



Thesis Adviser



Dean of the Graduate College

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TABLE OF CONTENTS

Chapter	Page
I. LITERATURE REVIEW.....	1
Introduction.....	1
Geographical Distribution and Prevalence...	2
Morphology and Culture.....	2
Genetic Variation.....	3
Pathology and Pathogenesis.....	4
Intracellular Survival.....	5
Surface Components of <u>Brucella</u>	7
Structure of LPS in Gram-negative Bacteria.	8
Biological Activity of LPS in Gram- negative Bacteria.....	9
Immunologic Properties of LPS in Gram- negative Bacteria.....	10
Lipopolysaccharide Extraction.....	11
Structure of <u>Brucella</u> LPS.....	11
Biological Activities of <u>Brucella</u> LPS.....	13
Immunologic Properties of <u>Brucella</u> LPS.....	15
Aim of the Study.....	17
II. MATERIALS AND METHODS.....	20
Cloning Strategy.....	20
Principle.....	20
The Cosmid Vector.....	23
Procedure.....	23
Preparation of Vector DNA.....	27
Preparation of <u>B. abortus</u> Chromosomal DNA..	27
Isolation of Genomic DNA.....	27
<u>Sau3A</u> Partial Digestion of Chromosomal DNA.....	28
Large Scale Partial Digest.....	29
Size Fractionation of Insert DNA.....	29
Ligation and Packaging of DNA.....	30
Ligation.....	30
Packaging.....	31
Titering the Cosmid Library.....	31
Screening of the Library.....	31
Screening with Polyclonal Antibody....	31
Screening by DNA-DNA Hybridization....	32
Screening with Monoclonal Antibody....	35

Chapter	Page
III. RESULTS	36
Preparation of Vector DNA.....	36
Preparation of <u>B. abortus</u> Chromosomal DNA..	36
Ligation of Vector and Insert DNA.....	39
Library Construction.....	39
Screening of the Library.....	40
Screening with Polyclonal Antibody....	40
Colony Lifts and Hybridization.....	43
Southern Transfer and Hybridization...	46
Digestion by Low-Frequency-Cutting	
Restriction Enzymes.....	53
Digestion with <u>Bam</u> HI.....	56
Hybridization with Labeled pWE15.....	56
Restriction Mapping of the Positive Clones.	59
Screening with Monoclonal Antibody.....	59
IV. DISCUSSION.....	67
LITERATURE CITED.....	76

LIST OF FIGURES

Figure	Page
1. Genomic DNA Cloning in Cosmid Vectors.....	22
2. The Cosmid Vector pWE15.....	25
3. 0.3% Agarose Gel Electrophoresis of Partial <u>Sau3A</u> Restriction Digestion of <u>B. abortus</u> DNA.....	38
4. Immunoblot of the Library on Nitrocellulose Filters Using Anti-Whole <u>Brucella</u> Antibody.....	42
5. Agarose Gel Electrophoresis of <u>EcoRI</u> Restriction Digestion of 16 Recombinant Clones.....	45
6. Colony Hybridization on Whatman 541 Paper Using UA2 Insert as a Probe.....	48
7. Gel and Blot for Southern Hybridization of <u>EcoRI</u> Digested Clones.....	50
8. Agarose Gel Electrophoresis of <u>EcoRI</u> Restriction Digestion of the 5 Positive Clones.....	52
9. Gel and Blot for Southern Hybridization of <u>HindIII</u> -digest of 5 Clones.....	55
10. Gel and Blot for Southern Hybridization of <u>BamHI</u> Digested Clones Probed with UA2.....	58
11. Gel and Blot for Southern Hybridization of <u>BamHI</u> Digested Clones Probed with pWE15.....	61
12. Proposed Restriction Map for the Positive Clones.....	63
13. Immunoblot of 20 Colonies on a Nitrocellulose Filter Using Anti- <u>Brucella</u> O-Antigen Monoclonal Antibody.....	66

CHAPTER I

LITERATURE REVIEW

Introduction

The genus Brucella includes three important intracellular bacterial pathogens that attack animals, from which they are transmitted to humans to cause undulant fever. One, known as Brucella melitensis causes malta fever in goats and sheep, a disease with prolonged fever, arthritis, reduced fertility in the herd and a tendency to abort. Another, B. abortus causes contagious abortion in cattle. This is characterized by a tendency to abort, retention of the placenta, sterility and death of newborn offspring. The third species, B. suis, causes contagious abortion in hogs (9).

Three other species, B. ovis, B. neotomae and B. canis have been described more recently, of which only the last appears to have any role in human disease (10).

Brucellosis is primarily a disease of animals. It spreads to man, but from the ecological point of view this is of little importance, for spread from human to human rarely, if ever, occurs. By contrast, infection of animals is an important factor in the ecology of Brucella, for infected animals readily infect one another, their illness

tends to be chronic, and they shed the organism into the environment, often for many years. Human infections are generally contracted in two ways: through ingestion of unpasteurized, infected milk or cheese or through contact with the tissues of infected animals. Human brucellosis may be an acute or relapsing febrile illness, a chronic illness, or a subclinical infection and the organisms persist in the mononuclear phagocytes (9).

All Brucellae are obligate parasites capable of causing acute or chronic illness or inapparent infection. The chronicity depends on their marked ability to multiply inside mononuclear phagocytes (18).

Geographical Distribution and Prevalence

The distribution of brucellosis in man and animals is worldwide and in all countries where it exists it is a serious economic problem (66). Moreover brucellosis presents an occupational hazard for farmers, shepherds, veterinarians, and laboratory workers (9).

Morphology and Culture

Brucellae are Gram-negative rods averaging in length from 0.6 to 1.5 μ and in width from 0.5 to 0.7 μ . They usually appear singly, sometimes in pairs or short chains (9).

Upon initial culture in vitro the organisms grow slowly on agar, the first colonies appearing usually after

48 hours and they show a smooth appearance with some rough forms as well. The addition of natural animal protein to the medium improves growth, and thiamine, nicotinic acid, pantothenic acid and biotin are required nutrients (62). The addition of 2.5 percent glucose or even more, the addition of erythritol (28), a substance which has been found in the placenta of some animals and may play some part in the localization of Brucellae in host tissues, favors growth (27).

Brucellae grow in the temperature range 20°C to 40°C, with an optimum of 37°C. Both B. melitensis and B. suis grow in atmospheric oxygen but for the primary isolation of B. abortus 5-10% CO₂ in the atmosphere is required (9).

Members of the genus Brucella vary in their sensitivity to certain dyes: thionine, basic fuchsin, methyl violet and pyronine are commonly used. B. melitensis grows freely in the presence of all four; B. abortus is sensitive to thionine but grows freely in the presence of the other three. B. suis grows in the presence of thionin but not of the other three (62).

Genetic Variation

When serially grown on laboratory media the virulent smooth (S) form of Brucella isolated from infected tissues tends to be replaced by rough (R) forms which are often less virulent and also exhibit less specific agglutination. This selection is due to the greater resistance of R cells

to alterations of the medium produced by the S cells, including accumulation of D-alanine and lowering of pO_2 (10). Nonsmooth variants can be observed through oblique light, they take up crystal violet, and show different phage susceptibilities than smooth variants. On electron microscopic examination, the lipopolysaccharide (LPS) of rough Brucella has a granular appearance, in contrast to the lamellar structures formed by Brucella smooth LPS (43).

Pathology and Pathogenesis

Following infection through the various routes, the organisms are first conveyed to the regional lymph nodes where the cell types involved are lymphocytes and macrophages. Here they may be held up and destroyed or, depending on the invasiveness of the organism, they may multiply, overcome the defenses of the lymphoid tissue and escape into the blood stream causing bacteremia (43). They are then carried in the blood stream around the body and localize mainly in the mononuclear phagocytic cells in the liver, spleen, bone marrow, lymph nodes and kidney. In the liver they may be seen inside phagocytes in the sinusoids and in the Kupffer cells. These cells containing the Brucellae tend to form aggregates or nodules, epithelioid or giant cells appear within the nodules and infiltrating lymphocytes appear around them. Similar granulomata form in the spleen, the lymph nodes, the bone marrow and the

kidney and are characteristic lesions of Brucella infection (9).

Brucellae typically invade the cells of their hosts (43). Inside the cells they may be protected from antibodies and from antibiotics as are Salmonella organisms. This intracellular habitat explains in part the chronicity of the disease in man and animals. Symptoms may be caused as the organisms escape from the mononuclear phagocytes into the bloodstream, or as the organisms disintegrate within the cells and release toxic substances (19, 43). This second mechanism may explain the pyrexia of brucellosis. It may also explain the Herxheimer type of reaction following administration of an antibiotic due to sudden release of endotoxin from the killed Brucellae, or when vaccine is injected into a hypersensitive person (19).

Intracellular Survival

Intracellular localization of Brucella organisms in mononuclear phagocytes has long been recognized. Brucellae were shown to establish infection and multiply in tissue culture in chick embryo fibroblasts and in guinea pig macrophages (18, 20). Under the cultural conditions used, the macrophages did not inhibit the parasite, but rather they provided a highly suitable environment for its growth (18). It was reported that, in tissue culture, smooth virulent B. abortus were able to multiply intracellularly while rough avirulent Brucellae either

failed to do so or multiplied only to a limited extent (60). Furthermore, smooth virulent Brucellae had little or no cytopathic effect on the infected host cell while rough avirulent Brucellae were rapidly destructive to the host cell. This may be related to the mechanism of virulence in the host. Smooth virulent Brucellae can multiply intracellularly with relatively little damage to the host cell and are thus somewhat protected from the humoral defenses whereas the rough Brucellae are periodically released and subjected to these defenses (17). The work of Smith and Fitzgeorge showed that cell wall preparations from B. abortus grown in vivo interfered with the killing of an avirulent strain by bovine phagocytes whereas the cytoplasmic contents did not, and they hypothesized that the immunogenic material in such cell wall preparations might contribute to the anti-bactericidal activity in bovine phagocytes (56).

In an attempt to characterize the mechanisms of intracellular survival exhibited by B. abortus, the bactericidal activity of guinea pig and human polymorphonuclear leucocytes against a smooth-intermediate strain and a rough strain was examined by Kreutzer et al. (31). Both strains were readily ingested, however no stimulation of the hexose monophosphate shunt was observed after ingestion of either strain. Degranulation as measured by electron microscopy, release of lysosomal enzymes and isolation of intact granules after incubation of polymorphonuclear leucocytes with

the smooth-intermediate strain of Brucella was significantly less compared with polymorphonuclear leukocytes incubated with extracellular bacteria. Guinea pig polymorphonuclear leukocytes incubated with B. abortus showed no respiratory burst (31, 55).

Thus, it appears that the cell surface of B. abortus enables it to escape destruction within the phagosome. Even though comparable levels of ingestion and degranulation were observed with both strains, the smooth strain was significantly more resistant to intracellular killing by polymorphonuclear leukocytes (54). Further studies to elucidate the relative importance of O_2 -dependent and -independent killing reactions present within human and bovine polymorphonuclear granule extracts showed that there was no O_2 -independent killing of either the smooth or rough strain. The O_2 -dependent brucellacidal activity of granule extracts was dependent on the concentration of myeloperoxidase units, H_2O_2 , and KI. The smooth strain was more resistant to O_2 -dependent killing by granule extracts than was the rough strain (54).

The only surface macromolecule present on the smooth strain and not detected on the rough strain was a phenol soluble LPS (30).

Surface Components of Brucella

Like the cells of other Gram-negative bacteria, Brucella cells are bound by an envelope consisting of an

inner or cytoplasmic membrane, an intermediate peptidoglycan layer and an outer membrane. The outer envelope is immunologically important because it is the part of the cell that is directly involved in cellular invasion and interacts with humoral host defense mechanisms. Peptidoglycan also plays a role. A peptidoglycan-containing fraction which is phenol-insoluble, extracted from B. melitensis, was shown to be immunogenic and induce protection in mice and rabbits without producing any allergic reactions (36).

Structure of LPS in Gram-Negative Bacteria

One of the major constituents of the outer membrane of Gram-negative bacteria is LPS. LPS consists of three distinct but covalently-linked classes of chemical compounds: lipid A, core polysaccharide and O-specific polysaccharide side chains (63).

The lipid A portion, which is embedded in the outer leaf of the outer membrane is a disaccharide of α -1, 6-linked glucosamines, substituted in the 1 and 4 positions by phosphate groups. The hydroxyl and amino groups are substituted by long chain fatty acids (7).

Attached to lipid A is a long and complex polysaccharide chain considered to have two parts. The inner portion is very similar within a genus, and contains two unusual sugars, 2-keto-3-deoxyoctonic acid (KDO) and a heptose,

both unique to bacteria. The other sugars in the core are more common in nature.

The O-specific side chain consists of variable numbers of repeating oligosaccharide units. These side chains often contain very unusual sugars and they vary remarkably in composition and arrangement. This portion of the LPS is the major serologic determinant of many Gram-negative bacteria (63).

Biological Activity of LPS in Gram-Negative Bacteria

Much information has accumulated over the past years relating the biological activity and the structure of LPS of Gram-negative bacteria and Enterobacteriaceae in particular. Crude preparations of LPS from smooth Brucella have been known to share some of the biological properties of enterobacterial LPS and to be unique in other respects (33).

LPS of all Gram-negative bacteria is toxic to mammals and many of the major symptoms of systemic Gram-negative infections can be reproduced with purified LPS. LPS is a very potent pyrogen and pyrogenicity is entirely attributable to the lipid portion of LPS (37). Endotoxin leads to the activation of at least four systems involved in the circulation and hemodynamics. These systems are interrelated and they can amplify each other (1). Activation of the complement system through both the classical and alter-

native pathways leads to platelet aggregation, thrombocytopenia, leucotaxis and potentiation of the inflammatory reaction (1). Activation of the coagulation system leads to disseminated intravascular clotting, bleeding due to consumption of clotting factors and platelets, tissue ischaemia and organ failure (39). Activation of the fibrinolytic system contributes to the bleeding tendencies (1). Activation of the bradykinin system causes hypotension, hypoxia, acidosis and shock (39). LPS also causes macrophage activation leading to release of interleukin 1 (endogenous pyrogen) and tumor necrosis factor (cachectin).

Immunologic Properties of LPS in Gram-Negative Bacteria

LPS modulates a spectrum of immunological responses both in vitro and in vivo. In vitro, LPS can initiate B lymphocyte proliferation resulting in the production of a large number of immunoglobulin synthesizing cells producing both IgM and IgG (7). T lymphocyte responses can be affected indirectly by LPS inducing cytokines from other cells. LPS also activates macrophages to release tumor necrosis factor (cachectin), interferon and interleukin 1 (endogenous pyrogen). Many of these immunologic activities are also manifested in vivo. LPS can act as an immunologic adjuvant for antibody formation and can modulate delayed-type hypersensitivity reactions (13). Furthermore, it was shown that LPS induces extravascular accumulation of

leukocytes and enhances nonspecific resistance to infection (7).

Lipopolysaccharide Extraction

The hot phenol-water method developed by Westphal et al. (64) has been widely employed for the extraction of LPS from Enterobacteriaceae. The LPS of most Gram-negative organisms is recovered from the aqueous phase in a relatively pure form. Brucella endotoxin differs from other Gram-negative endotoxins in that it is recovered in the phenol phase rather than the aqueous phase. This was first described by Redfearn (53) with phenol-killed smooth B. abortus and B. melitensis and has since been confirmed by others (11, 24). Leong et al. (33) thoroughly documented that this phenol-phase extracted B. abortus LPS shares many of the physiological, structural and chemical properties of the enterobacterial LPS recovered from the aqueous phase. The partitioning of Brucella LPS into the phenol phase is not unique. Phenol-soluble endotoxins have been isolated from other Gram-negative bacteria such as Citrobacter freundii (52), a heptose-lacking mutant of Salmonella minnesota (38), and Yersinia enterocolitica type 0:9 (21).

Structure of Brucella LPS

Antigenic phenol-soluble LPS isolated from B. abortus was shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, controlled hydrolysis, periodate oxida-

tion, methylation and nuclear magnetic resonance studies to be an S-type LPS which could be cleaved to yield lipid A, core, and an O-chain polysaccharide (8). Aldoheptose, which is a common LPS core constituent, was not detected (8, 48) giving evidence that the core region forms a minor component (8).

The O-chain polysaccharide was identified to be an unbranched linear homopolymer of 1,2-linked perosamine (4, 6-dideoxy-4-formamido- α -D-mannopyranose units) with an average chain length of 96-100 glycosyl units (8).

The lipid A component is composed of 2-amino-2-deoxy-D-glucose (10.1%), phosphate (5.9%), n-tetradecanoic acid (12%), n-hexadecanoic acid (33%), n-octadecanoic acid (15%), 3-hydroxytetradecanoic acid (27%) and 3-hydroxyhexadecanoic acid (4%) (8) i.e. only 5 major fatty acids. B-hydroxymyristic acid, a common marker of enteric LPS, was not detected (29, 48).

Purified Brucella LPS preparations contain approximately 6% residual protein tightly bound to the lipid A moiety. This protein could not be removed from the smooth LPS by gel filtration in the presence of detergent and the chaotropic agent NaI, by ultracentrifugation or in SDS-PAGE after boiling in the presence of 2-mercaptoethanol suggesting a covalent linkage between the protein and the smooth LPS. This covalently bound protein may account for the preferential solubility of Brucella smooth LPS in the phe-

nol phase (23, 48). In contrast to the S-LPS, protein was not found to be bound to R-LPS (23).

The unique fatty acid composition of Brucella lipid A may be responsible for some of the distinctive biological activities of Brucella LPS (46, 47, 49).

Biological Activities of Brucella LPS

Crude endotoxin from smooth Brucella presents both similarities and differences when compared with LPS from Enterobacteriaceae. Some of the similarities were documented through the work of Leong et al. (33). They showed that Brucella endotoxins are heat-stable and resistant to pronase digestion. Electron micrographs of Brucella endotoxins showed forms which were morphologically indistinguishable from those of enterobacterial endotoxins. A 25 mg dose of Brucella LPS, administered intravenously, induced severe leukopenia with subsequent leukocytosis in mice as occurs with LPS from Enterobacteriaceae (21).

Other work has demonstrated that Brucella endotoxins are potent antigens and are capable of inhibiting the agglutination of smooth Brucella cells by homologous O-antisera (11). They are toxic for mice although the toxicity is less than that caused by enterobacterial LPS (3). Other similarities include induction of reduction in serum iron in rats (4). Cross-tolerance experiments in mice showed that pretreatment with B. abortus endotoxin lessened

the hypoferremia produced by challenge with Escherichia coli endotoxin. Reciprocal cross-tolerance to the hypoferremic capacity of B. abortus endotoxin could be established by pretreatment of mice with E. coli endotoxin (33). Similarities were also found in the Limulus lysate assay for endotoxin. Comparable amounts of endotoxin were required to cause clotting of the Limulus lysate; the standard dose for Klebsiella endotoxin to cause clotting is 0.25 nanograms, and one nanogram of the phenol fraction of Brucella cells caused clotting. One hundred nanograms of the aqueous fraction were required for similar clotting (23, 50).

Further differences in biological activity between Brucella LPS and enterobacterial LPS have also been described. Brucella LPS was found to be nonpyrogenic for rabbits and nonlethal for chick embryos (33), and did not increase the susceptibility of mice to histamine hypersensitivity when compared to LPS of four Salmonella species (50). These findings are consistent with the hypothesis that the mediation of its toxicity may be unique. It is possible that the unique fatty acid composition of Brucella lipid A is responsible for some of the distinctive biological activities of Brucella LPS. The strongly adherent protein of Brucella LPS may also contribute to these distinctive biological activities (47).

Immunologic Properties of Brucella LPS

Similar to enterobacterial LPS, isolated Brucella S- and R-LPS and Brucella lipid A were shown to activate the complement cascade (47). Similarities were also found in induction of interferons after intravenous injection of B. abortus LPS into mice (26). Spellman and Reed (59) studied the immunogenic and mitogenic properties of B. abortus LPS using normal and congenitally athymic mice. C3H/HeJ mice, known to have a genetically controlled low response to the endotoxins of E. coli and Salmonella, were included in these studies to determine if this genetic defect would also limit their response to B. abortus LPS. They concluded that B. abortus LPS, like E. coli LPS, appears to be a thymus independent antigen with respect to evoking T-independent 2-mercaptoethanol-sensitive primary antibody responses in mice. However, unlike E. coli LPS, which elicits a postimmunization antibody response in mice detectable by day 3 and waning by days 10 to 14 (44), B. abortus LPS elicits antibody detectable by day 4 and persisting to at least day 30.

Upon a secondary challenge, crude B. abortus LPS preparations elicit both 2-mercaptoethanol-sensitive and 2-mercaptoethanol resistant antibodies in mice whereas E. coli LPS elicits only a heightened 2-mercaptoethanol-sensitive response. It was concluded that T cells are apparently required for an optimum secondary response to B. abortus LPS (44).

Brucella endotoxin differs from E. coli endotoxin in at least one other respect. The C3H/HeJ mouse, an endotoxin insensitive strain, showed antibody responses comparable to normal mouse responses to B. abortus LPS. These conflicting results are probably due to the protein that does not dissociate from Brucella LPS (59).

It was also demonstrated that B. abortus LPS is mitogenic for spleen cells from normal and nude mice and C3H/HeJ mice, but not for thymus cells from normal or C3H/HeJ mice, suggesting that they are potent B cell mitogens (59). Spellman and Reed postulated that the high protein content of their B. abortus LPS preparation may be important in the mitogenic capacity of the endotoxin. Other workers confirmed the mitogenic activation by Brucella LPS of spleen cells from endotoxin-resistant C3H/HeJ mice and were able to reproduce it by lipid A (47). Moreno and Berman also supported the hypothesis that B lymphocytes are probably responding to the mitogenic stimulus induced by the unique lipid moiety of Brucella LPS, i.e., lipid A is responsible for mitogenicity. They also showed that highly purified B. abortus rough LPS, which lacks the O-polysaccharide antigen was mitogenic. Mitogenicity was not inhibited by polymyxin B, and amino acid analysis showed no binding of polymyxin B to Brucella LPS under conditions in which mitogenicity of E. coli LPS was inhibited (47). It is known that polymyxin B binds to the lipid A-2-keto-3 deoxyoctonate moiety of LPS in E. coli and

Salmonella. So we can conclude that Brucella lipid A is different from E. coli Lipid A regarding its polymyxin B binding site.

Unlike enterobacterial LPS, which induces the production of predominantly IgM antibody, B. abortus LPS stimulates the production of IgM and high levels of IgG in both responsive and nonresponsive strains of mice and in athymic mice (49). The demonstration that B. abortus LPS was an adjuvant in responsive mice but not in C3H/HeJ mice clearly separates its capacity to act as a B cell mitogen and polyclonal activator from adjuvanticity (49).

The fact that euthymic mice made greater secondary IgG responses than nude mice to both S-LPS and R-LPS of B. abortus suggests that differentiated T cells in the euthymic mice participated in the responses. It is possible that B. abortus LPS, in addition to delivering the antigenic signal, is able to trigger the signals for both proliferation of B cells and the switch from IgM to IgG production. The action of B. abortus LPS on macrophages suggests that one or both of these signals may be mediated through activation of these cells (49).

Aim of the Study

LPS of Brucella is one of the surface components of the cell and therefore interacts directly with the host defense mechanisms.

Recent studies using the murine model system have provided evidence through both active (65), and passive (34, 45) immunization that antibodies against the O-antigen play an important role in protection.

Winter et al. (65) have shown that a single immunization of mice with a complex of porin and S-LPS extracted from a virulent Brucella strain provided significant protection against challenge with the same strain, equivalent to that achieved by vaccination with the living attenuated B. abortus strain 19, the vaccine strain. This protective vaccine caused the formation of increased concentration of circulating O-polysaccharide specific antibodies, whereas antibodies specific for moieties other than O-polysaccharide, as lipid A or core or porin, were found in very low concentration in sera of the immunized mice.

Another study by Montaraz et al. (45), showed that mice injected with monoclonal antibodies specific for the O-polysaccharide of B. abortus were protected against challenge with virulent B. abortus cells and they had viable counts of Brucella in the spleen and liver which were significantly below those in the control groups.

These results provide evidence that the O-polysaccharide may constitute an essential component of an effective subunit vaccine against B. abortus and that O-polysaccharide specific antibodies play an important role in protective immunity in brucellosis.

Hence, it is the specific aim of this study to construct a B. abortus cosmid library in E. coli and to localize the genes coding for LPS production. The use of a cosmid vector allows for cloning large fragments (30-40 Kb) of Brucella DNA. The library will be screened with polyclonal antibodies against Brucella and monoclonal antibodies against LPS. A second method of screening is to use a cloned 1.5 Kb Brucella insert from a λ gt11 library to probe the cosmid library for clones carrying larger sequences coding for Brucella LPS components. The 1.5 Kb insert has been shown to react with a monoclonal antibody prepared against purified Brucella LPS.

Because Brucella is a class III pathogen, direct handling of the organism is restricted and requires extreme care. Cloning of Brucella genes in a nonpathogenic organism like E. coli will allow for studying the Brucella organism and its genetic structure without the dangers of working with B. abortus directly. Cloning of LPS genes would allow the production of large amounts of pure LPS. Thus, the immunogenic role of LPS alone, free of adherent protein, could be determined. Also the cloned product could be evaluated as a potential subunit vaccine.

CHAPTER II

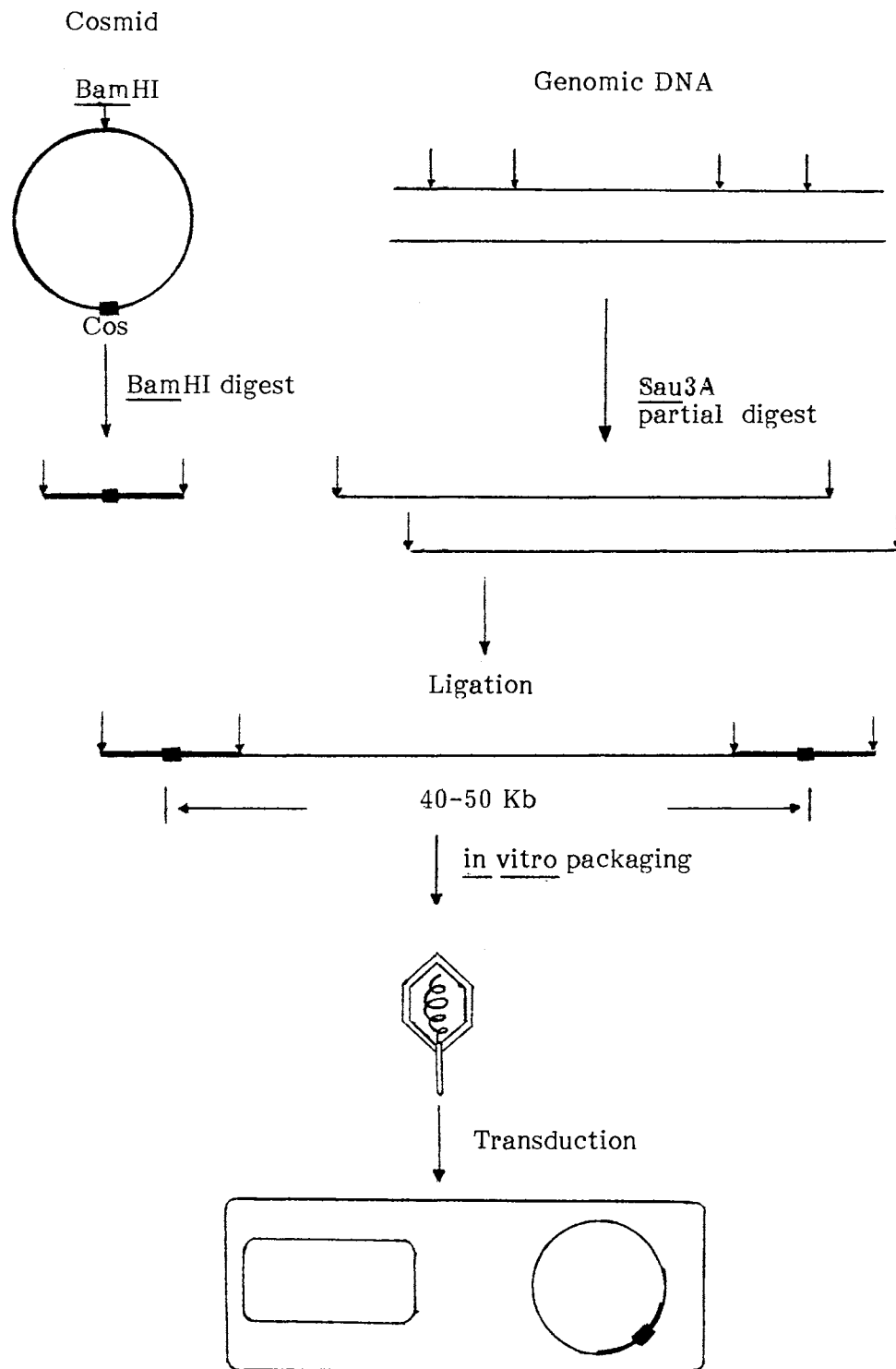
MATERIALS AND METHODS

Cloning Strategy

Principle

The structural and functional analysis of complex genomes often requires the isolation and mapping of large regions of DNA. Cosmid vectors are valuable tools in this analysis because they can accommodate genomic DNA fragments ranging in size from 30-45 Kb. The basic principle for the cosmid cloning technique is shown in Figure 1 (12). A ligation mixture was set up containing the cosmid vector pWE15 (14) and B. abortus DNA that have been digested with a restriction endonuclease. Among the products of ligation were B. abortus DNA linked to cosmid molecules such that both the cos sites were in the same orientation and all the plasmid information is between the 2 cos sites. The ligated molecules were packaged in vitro into lambda phage particles. E. coli were infected by lambda particles, the recombinant DNA was injected into the cell to circularize via the cohesive ends. The cosmid can replicate as a plasmid and is capable of expression of an ampicillin resistance gene.

Figure 1. Genomic DNA Cloning in Cosmid Vectors



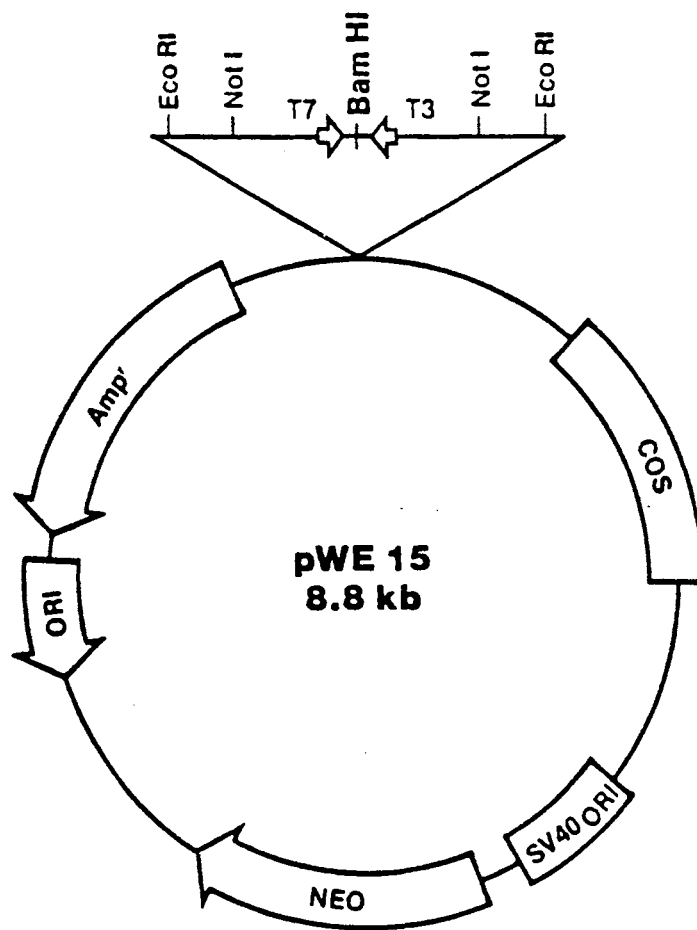
The Cosmid Vector

The vector used here was the 8.8 Kb cosmid pWE15 (Figure 2). It contains the *colE1* origin of replication (22), a lambda bacteriophage *cos* site, an ampicillin resistance gene, and bacteriophage T3 and T7 promoter sequences. By using the cosmid DNA containing a genomic insert as a template for either T3 or T7 RNA polymerase, directional "walking" probes can be synthesized and used to rescreen the library to isolate overlapping clones. These promoters also allow the restriction map of the recombinant cosmid to be easily determined (14). In addition, recognition sequences for the restriction enzyme NotI have been placed near the BamHI cloning site. NotI is infrequently represented in the mammalian genome, which allows its use to excise most genomic inserts as a single large restriction fragment. This feature can be useful for the in vitro reconstruction of large genes, which are greater than the cloning capacity of the cosmid (14).

Procedure

The cosmid DNA vector containing the lambda cos site and a selectable ampicillin resistance marker was cut with BamHI at the unique site. The protruding single stranded termini of the resulting molecule were made incapable of ligation to each other by alkaline phosphatase treatment.

Figure 2. The Cosmid Vector pWE15



High molecular weight Brucella abortus DNA (>150 Kb) was partially digested with Sau3A to yield large fragments (30-40 Kb) with compatible sticky ends. A series of test partial digests was done to determine the ideal conditions for obtaining the desired insert size range. The partial digestion was followed by size fractionation on a sucrose gradient. Vector and insert were then ligated yielding high molecular weight concatemers. To minimize ligation of noncontiguous Brucella DNA segments, a 10-fold molar excess of cosmid over insert was used in the ligation mix. Packaging the recombinant molecules into phage particles selects for recombinants with a total size (cosmid plus chromosomal DNA) of 38-52 Kb (40). The bacteriophage particles were subsequently transduced into recombination deficient E. coli to reduce recombination and increase stability of the cosmid DNA. The recombinant cosmid DNA is then propagated in E. coli as a large plasmid distinct from the E. coli chromosome.

Screening of the library was done with polyclonal antibodies against whole Brucella cells. Rescreening of the positive colonies was done with a monoclonal antibody specific for LPS. The library was also screened by DNA-DNA hybridization with a 1.5 Kb Brucella insert which has been previously isolated from a λ gt11 library, and has been shown to react with a monoclonal antibody prepared against purified LPS. Positive hybridization leads to the identification of cosmid clones carrying larger sequences of LPS.

Preparation of Vector DNA

The vector pWE15 (Stratagene, La Jolla, CA) was digested with the restriction enzyme BamHI (Bethesda Research Laboratories, Gaithersburg, MD), the DNA extracted with phenol/chloroform/isoamyl alcohol and dephosphorylated with calf intestinal alkaline phosphatase (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). This was performed by a modification of the method of Dilella and Woo (12). The modifications included no reextraction of the organic phase, ethanol precipitation at -20°C rather than on dry ice and omission of washing of the pellet with 70% ethanol. Alkaline phosphatase treatment was followed by test ligation of the dephosphorylated vector to test the efficiency of the phosphatase reaction as well as integrity of the BamHI cohesive ends. The test ligation was carried out as described by Dilella and Woo (12).

Preparation of B. abortus

Chromosomal DNA

Isolation of Genomic DNA

The quality of genomic DNA that is used to make the cosmid library is the most critical component for the success of library construction. Because large fragments are required, the genomic DNA must be very large (150-200 Kb) before digestion. DNA from B. abortus S19 was prepared according to the method of Bates (5) which avoids all vig-

orous pipetting and precipitation steps which may cause mechanical shearing of the DNA. Also the phenol and chloroform extractions were done with a large surface area between the aqueous and organic phases to avoid vigorous mixing during extraction. The size of the genomic DNA was checked by electrophoresis through a 0.3% agarose gel. The best resolution with this gel system was obtained by running the gel at 1-2 V/cm for 18-20 hours at 4°C. T4 DNA (160 Kb) and lambda DNA (50 Kb) (New England Biolabs, Beverly, MA) were used as size markers. Genomic DNA that migrated with T4 DNA was considered to be of good quality.

Sau 3A Partial Digestion of Chromosomal DNA

In order to clone into the BamHI site of the pWE cosmid vector, B. abortus DNA was partially digested with Sau3A (New England Biolabs, Beverly, MA). A series of test partial digests of B. abortus was set up as described by Bates (5) to determine the ideal conditions for obtaining the desired insert size range which is 30-43 Kb. This was done by removing aliquots from the digestion reaction after 5, 10, 15 and 20 minutes of digestion. These aliquots were analyzed on a 0.3% agarose gel. Various DNA size markers were used: uncut lambda DNA (48 Kb), lambda cut with XhoI (33 and 15 Kb) (New England Biolabs, Beverly, MA) and lambda cut with HindIII (28 and 23 Kb) (Bethesda Research Laboratories, Gaithersburg, MD). The gel was run at 2 V/cm

for 16 hours to get good separation and accurate sizing. The gel lanes were compared to choose the time point which produces the most intense staining in the 30-43 Kb range. The optimum conditions were determined to be approximately 0.1 unit of Sau3A/μg of B. abortus DNA for 5 minutes.

According to Maniatis et al. (40) it was found that the best conditions based on the cloning efficiency is usually a slightly shorter digestion time than the time which gives the most intense 30-43 Kb staining. This gives an optimum sequence representation of molecules in this size range.

Large Scale Partial Digest

After the optimal digestion time was determined, the test digest conditions were scaled up to digest 300 μg of chromosomal DNA. An aliquot from the reaction was electrophoresed on a 0.3% gel to insure appropriate size distribution. After removal of the enzyme by phenol/chloroform extraction, the DNA was precipitated with ethanol. At this point the DNA should only be about 50 Kb in length and shearing is not a major problem. Precipitation was followed by gentle suspension of DNA in 10 mM Tris HCl, 1 mM EDTA, pH 8.0 (TE buffer).

Size Fractionation of Insert DNA

Sucrose gradients were used to separate the required size fragments of B. abortus DNA by a modification of the

method of Maniatis et al. (40). Two 11 ml gradients were used in an SW-41 Beckman rotor. The gradients were poured using a gradient maker from 10% and 40% sucrose solutions. The partially digested DNA was layered onto the gradients and centrifuged at 30,000 rpm at 10°C for 12 hours in a model L5-50 Beckman ultracentrifuge. The gradients were fractionated by introducing a pusher solution to the bottom of the gradients (50% sucrose plus phenol red) using a fraction collector. The fractions containing the correct size range were determined by electrophoresis on a 0.3% agarose gel. The DNA of appropriate size was located in fractions 15-20. The fractions containing the DNA of the desired size were pooled, the DNA precipitated and resuspended in TE buffer.

Ligation and Packaging of DNA

Ligation

Vector and insert were ligated using T4 DNA ligase (International Biotechnologies, Inc., New Haven, CT) in a reaction where the total concentration of DNA in the ligation was 225 µg/ml and the vector was present in a 10-fold molar excess (5). At the end of the reaction an aliquot was removed and checked along with an unligated aliquot by electrophoresis on a 0.3% agarose gel. If ligation is successful the chromosomal DNA should be converted to high molecular weight concatemers. This should not be too

apparent, however, since a 10-fold molar excess of vector has been used.

Packaging

The ligation reaction was packaged in vitro into Giga-pack XL packaging extracts (Stratagene, La Jolla, CA) according to the manufacturers specifications.

Titering the Cosmid Library

The recombinant library was titered on E. coli AG1 cells plated on LB plates with ampicillin (25 µg/ml) according to the protocol of Maniatis et al. (40). The titer of the library was 1×10^6 recombinants per microgram of size-fractionated chromosomal DNA.

Screening of the Library

Screening with Polyclonal Antibody

Screening of the library was done with polyclonal antibody against B. abortus S19 produced in rabbits after injection with heat-killed organisms. The antibody was adsorbed with E. coli AG1 cells prior to use.

The recombinant library was plated on E. coli AG1 host cells. Following an overnight incubation at 37°C, the plates were overlaid with sterile nitrocellulose filters (Schleicher and Schuell, Inc., Keene, NH) to form replicas. Replica filters were exposed to chloroform vapour, blocked with 5% non-fat dry milk in 50 mM Tris-HCl, 150 mM NaCl, pH

7.5 (TBS) for 20 minutes and then washed 3 times in TBS. The filters were then incubated with the anti-Brucella antibody, washed again 3 times with TBS and incubated with a protein A-horseradish peroxidase conjugate (Sigma Chemical Company, St. Louis, MO). The antigen-antibody complexes were visualized using 4-chloro-1-naphthol as substrate. Several hundred colonies were shown to be positive with the anti-whole Brucella antibody. Heat-killed Brucella cells and AG1 cells were used as positive and negative controls respectively.

Screening by DNA-DNA Hybridization

Colony Lifts and Hybridization. The method of Taub and Thompson (61) was used for the purification of DNA on filter paper from hundreds of recombinant colonies simultaneously. Briefly, the colonies were grown at 37°C on LB agar plates with ampicillin. The colonies were lifted onto sterile Whatman 541 paper which was then incubated overnight on plates of LB agar containing chloramphenicol (250 µg/ml) to amplify the cosmids. After treatment with NaOH, enzymatic digestion and organic solvent extraction of the DNA bound to the paper, the filters were ready for hybridization.

Probe. The recombinant colonies were probed with a cloned 1.5 Kb Brucella insert. The insert had been previously identified from a λgt11 library of B. abortus S19 DNA and subcloned into the plasmid pUC19 and was shown

to react with a monoclonal antibody prepared against purified LPS. The 1.5 Kb insert was isolated by EcoRI (Bethesda Research Laboratories, Gaithersburg, MD) digestion of pUC19, running the digested plasmid DNA on low melting point agarose gel and extraction of the insert from the agarose by using the Nucleic Acid Chromatography System (NACS column, Bethesda Research Laboratories, Inc., Gaithersburg, MD). The probe was labeled with photobiotin (Vector Laboratories, Inc., Burlingame, CA) using the method of Forster et al. (16). The labeling was determined by detection of the spotted probe onto nitrocellulose paper.

The hybridization between the biotinylated probe and the recombinant colonies lifted on filter paper was performed by the method of Maniatis et al. (40) as modified by Leary et al. (32). The standard hybridization conditions were modified for use with the biotin-labeled probe due to the lower melting temperature of the probe-target hybrid. The modifications included using higher probe concentration, denaturation of the biotinylated probe by boiling rather than alkali treatment, reduction of the concentration of formamide from 50% to 45% in the prehybridization/hybridization buffer and incubation of the reaction at 42°C for the desired time period. The probe was used at a concentration of 500 µg/ml of buffer.

The hybridization was detected using the DNA detection system (Bethesda Research Laboratories, Inc., Gaithersburg,

MD) according to the manufacturer's instructions. The system utilizes a streptavidin-alkaline phosphatase conjugate to identify the positive clones which show a blue color. As negative and positive controls, an E. coli AG1 colony and a pUC19 colony with insert, were included in the screening. Twenty colonies were found to hybridize with the Brucella insert.

Preparation of Recombinant Cosmid DNA from Minilysates. The twenty colonies showing a hybridization signal were picked from the library and plated out on an LB ampicillin plate. Cosmid DNA was isolated from the recombinant clones from 3.0 ml mini-cultures by a modification of the alkaline lysis method of Birnboim and Doly (6) as described by Maniatis et al. (40).

An aliquot of the DNA from each clone was digested with EcoRI restriction endonuclease and the resulting fragments were analyzed by agarose gel electrophoresis.

Southern Transfer and Hybridization. DNA from the twenty positive clones digested with EcoRI and separated by agarose gel electrophoresis was transferred to nitrocellulose filters by the method of Southern (57) as described by Maniatis et al. (40). The filters were then hybridized with the same biotinylated probe that was used in colony blotting, and using the same hybridization conditions as before. pWE15 with no insert, digested with EcoRI was used in the Southern blots as a negative control and

pUC19 with the 1.5 Kb insert was used as a positive control. Five clones were found to show hybridization with the Brucella insert.

Screening with Monoclonal Antibody

The 20 clones shown to be positive upon colony hybridization were tested with an anti-Brucella O-antigen monoclonal antibody prepared in tissue culture cells. The monoclonal antibody, a gift from Dr. Marshall Phillips, was prepared at the National Animal Disease Center in Iowa. The procedure used for testing was the same as that used with polyclonal antibody.

CHAPTER III

RESULTS

Preparation of Vector DNA

The vector pWE15 was digested with the restriction enzyme BamHI and was shown by agarose gel electrophoresis to be in the linear form. The vector was dephosphorylated with calf intestinal alkaline phosphatase to prevent self ligation of the linearized vector DNA at the BamHI sites. Upon test ligation of the vector, one linear 8.5 Kb cosmid band was seen both before and after ligation, showing that the phosphatase treatment was efficient.

Preparation of B. abortus

Chromosomal DNA

Chromosomal DNA was isolated from B. abortus according to the method of Bates (5) and the DNA was analysed by electrophoresis through a 0.3% agarose gel. B. abortus DNA comigrated with a 160 Kb T4 DNA sample indicating that it was of suitable size before digestion. A series of test partial digests of the chromosomal DNA with Sau3A was set up as described before and aliquots were analysed on a 0.3% agarose gel as shown in Figure 3. The optimum conditions

Figure 3. 0.3% Agarose Gel Electrophoresis of Partial Sau3A Restriction Digestion of B. abortus DNA

Lane a: HindIII-digested lambda DNA size marker

Lane b: XhoI-digested lambda DNA size marker (33 and 15 Kb bands)

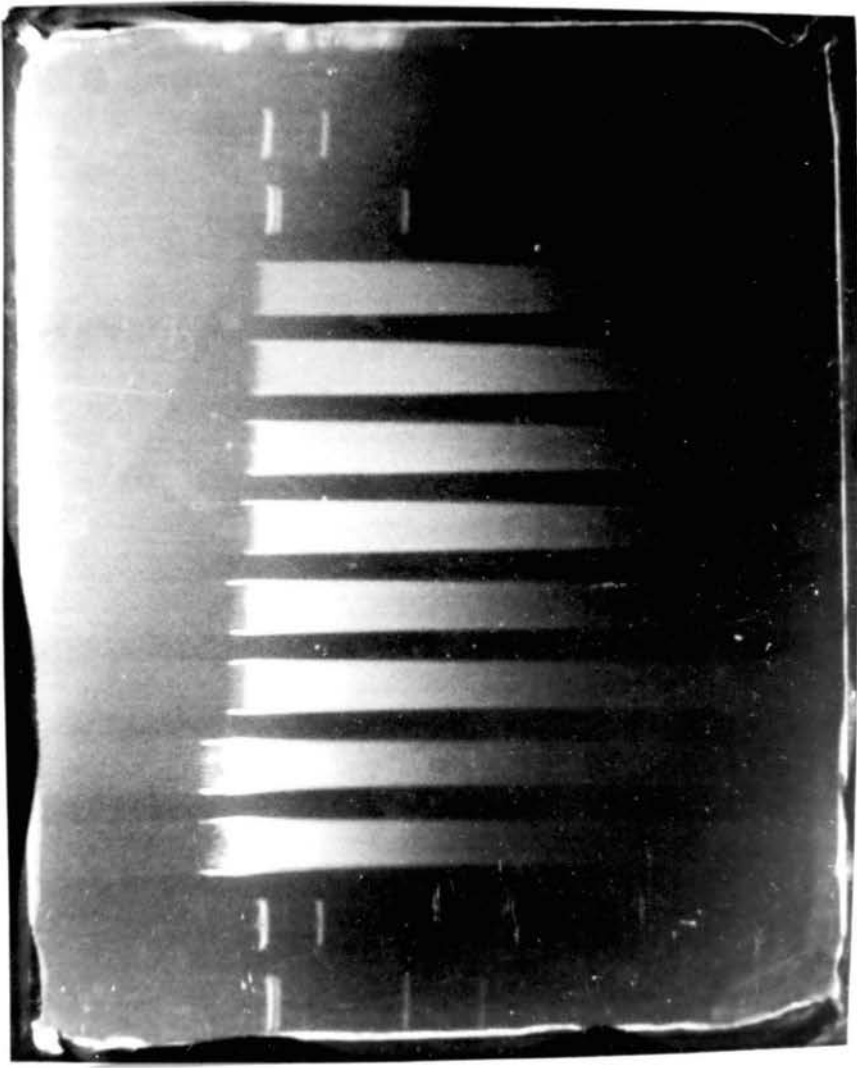
Lane c: B. abortus DNA after 5 minutes of digestion

Lane d: B. abortus after 10 minutes of digestion

Lane e: B. abortus after 15 minutes of digestion

Lane f: B. abortus after 20 minutes of digestion

a b c c d d e e f f a b



were determined to be approximately 0.1 unit of Sau3A per microgram of B. abortus DNA for 5 minutes. The test digest conditions were scaled up to digest 300 μg of chromosomal DNA. The required size fragments were separated using sucrose gradient centrifugation.

Ligation of Vector and Insert DNA

Vector and insert DNA were ligated in a reaction where the total concentration of DNA in the ligation was 225 $\mu\text{g/ml}$ and the vector was present in a 10-fold molar excess. On agarose gel electrophoresis the ligation was not too apparent since a 10-fold molar excess of vector had been used. Also, the insert DNA migrates in the nonlinear region of the 0.7% gel, so it was not possible to detect any differences in the ligated and unligated insert DNA band.

Library Construction

A B. abortus cosmid library containing 30-40 Kb fragments of the B. abortus genome was constructed in E. coli. Recombinant cosmid molecules were packaged in vitro into lambda bacteriophage particles and subsequently transduced into E. coli AG1 cells. The packaging efficiency obtained was 1×10^6 recombinants per microgram of size-fractionated chromosomal DNA. Plating out of the library on LB plates containing ampicillin resulted in obtaining 1.4×10^4 ampi-

cillin-resistant colonies. Because the packaging capacity of bacteriophage lambda particles is ~37-50 Kb of total DNA, all of the ampicillin-resistant colonies should contain inserts. The size of the Brucella genome is 4.7×10^6 base pairs (2) and the number of recombinants required for representation of the whole Brucella genome is 700 to 800 colonies. The number of ampicillin-resistant recombinants obtained is >15 times the number of colonies needed to represent all Brucella sequences. The titer of the frozen library was 5×10^{10} cfu/ml.

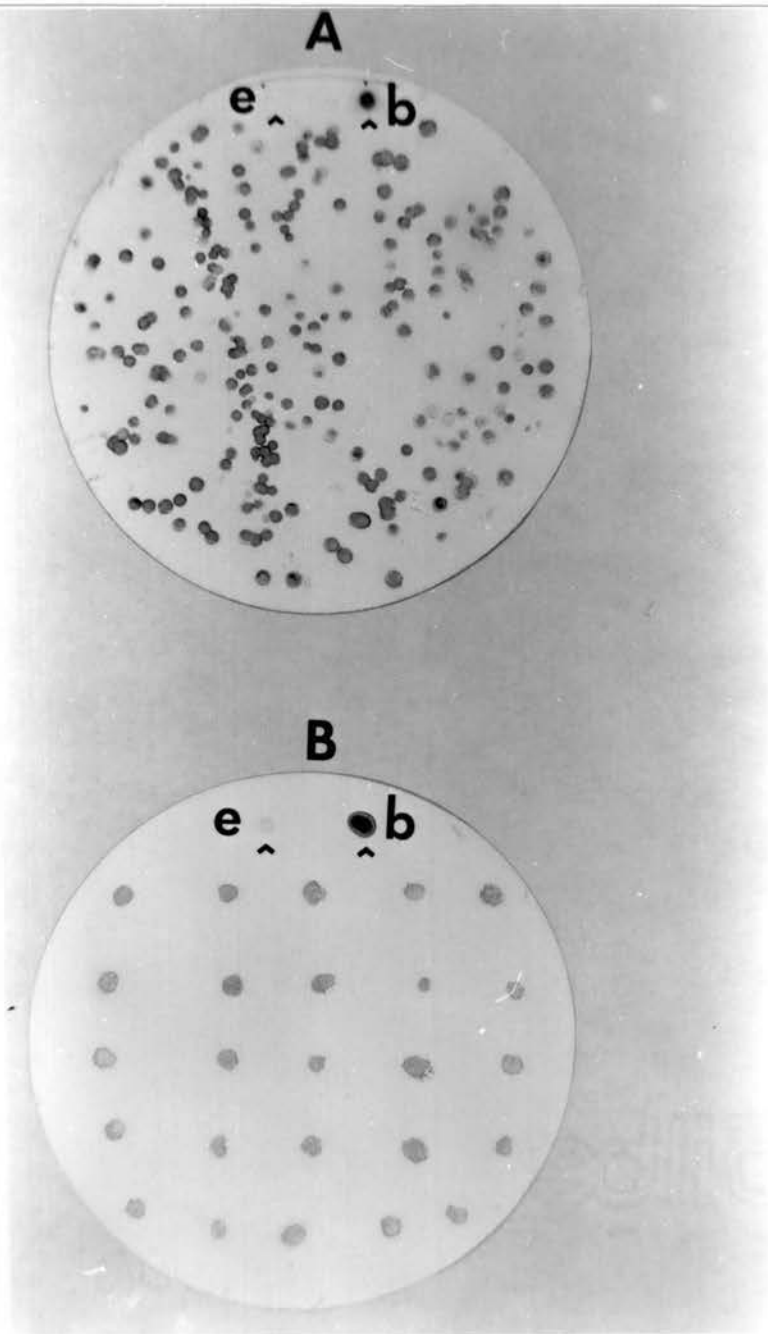
Screening of the Library

Screening with Polyclonal Antibody

The antibody used to screen the library was a rabbit source polyclonal anti-whole B. abortus S19 antibody. The recombinant library was plated on LB plus ampicillin medium and the resultant colonies were lifted onto nitrocellulose filters. Control organisms, E. coli and Brucella were spotted on the nitrocellulose filters after the lift. Of the 1000 colonies screened more than 80% were shown to be positive, i.e. showed a positive color reaction.

Randomly, 25 positive colonies were picked and transferred to a new LB ampicillin plate and once again screened with the same antibody. The results of the antibody screening are shown in Figure 4.

Figure 4. Immunoblot of the Library on Nitrocellulose
Filters Using Anti-Whole Brucella Antibody
Filter A: one of the filters from the
library showing positive colonies
Filter B: some of the positive colonies
selected from Filter A and retested with
the same antibody
e: E. coli cells as negative control
b: Brucella cells as positive control



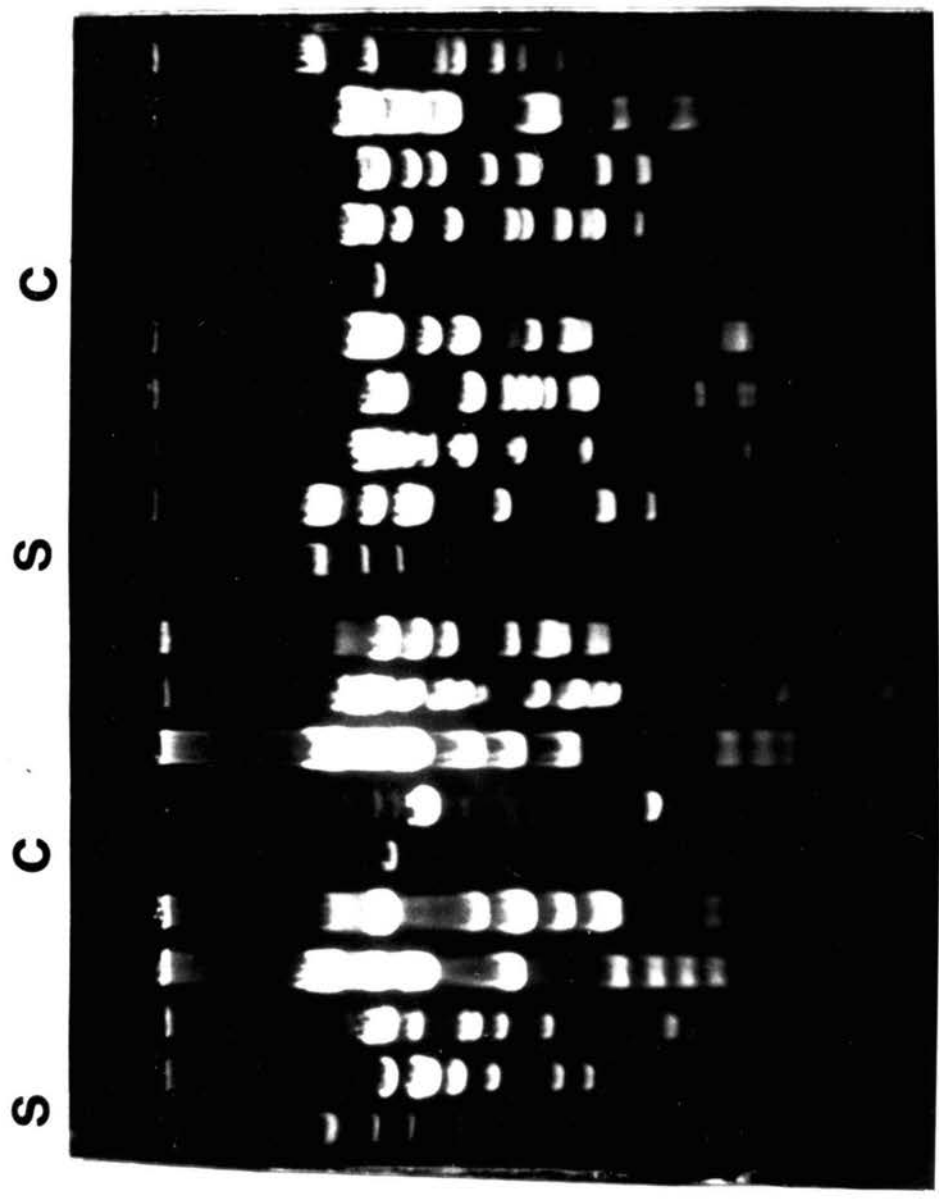
Preparation of Recombinant DNA from Minilysates: DNA was extracted from 16 randomly selected positive colonies and digested with EcoRI to ensure that they contained inserts. The vector pWE15 has 2 EcoRI sites that are very close together in the cloning region and showed one major linear band upon EcoRI digestion. Digestion of DNA from the positive colonies with the same enzyme showed clearly that they had insert DNA as shown in Figure 5.

Colony Lifts and Hybridization

Because antibody screening showed hundreds of recombinant colonies, a more specific method, DNA-DNA hybridization, was the next method used for screening.

The probe used was a previously cloned 1.5 Kb Brucella insert (UA2) from a λ gt11 library that was subcloned into the unique EcoRI site of the plasmid pUC19. The UA2 insert had been shown to react with a monoclonal antibody prepared against purified Brucella LPS. In this study the UA2 insert was isolated, labeled with photobiotin and used to probe the library which was replica filtered onto paper. Hybridization of the colonies on nitrocellulose filters gave very high background due to nonspecific binding. The use of Whatman 541 paper instead of nitrocellulose gave very clear results. Twenty colonies were identified that showed hybridization with the UA2 insert. Included in the screening were an E. coli AG1 colony as a negative control

Figure 5. Agarose Gel Electrophoresis of EcoRI
Restriction Digestion of 16 Recombinant
Clones
Lane S: HindIII-digested lambda DNA
size marker
Lane C: pWE15 DNA digested with EcoRI
All other lanes: recombinant clones
digested with EcoRI



100% (100) (100) (100)

and a pUC19 colony with UA2 insert as a positive control. Figure 6 shows the result of the colony hybridization.

Southern Transfer and Hybridization

EcoRI-digestion and Southern Hybridization: To further characterize the positive clones regarding the size of the specific DNA fragments of interest that are hybridizing with the UA2 insert, Southern blots were performed. DNA from the 20 recombinant clones was EcoRI digested, the fragments analysed by agarose gel electrophoresis and blotted onto nitrocellulose. Southern hybridization was performed using biotinylated UA2 as a probe. It had been shown before that UA2 has no internal EcoRI sites. The results of the hybridization showed homology with 5 of the screened clones as shown in Figure 7.

EcoRI-digested DNA from the 5 positive clones was analysed by agarose gel electrophoresis along with a 1Kb DNA ladder to determine the size of the inserts and of the hybridizing fragments (Figure 8).

Clone 1 showed a 9.5 Kb EcoRI fragment which hybridized with UA2. This clone has 9 EcoRI fragments plus one pWE15 EcoRI fragment. The size of the insert was determined to be 30.5 Kb.

Clone 2 showed a 2.2 Kb EcoRI fragment which hybridized with UA2. The clone has 15 EcoRI fragments plus one pWE15 EcoRI fragment. The insert size in this clone is 32.8 Kb.

Figure 6. Colony Hybridization on Whatman 541 Paper Using
UA2 Insert as a Probe
Filter A: one of the filters from the
library showing a positive colony
Filter B: the 20 positive colonies selected
from the colony hybridization and retested by
hybridization with the same probe
p: colony of pUC19 with insert as a positive
control
e: E. coli colony as a negative control
r: positive colonies

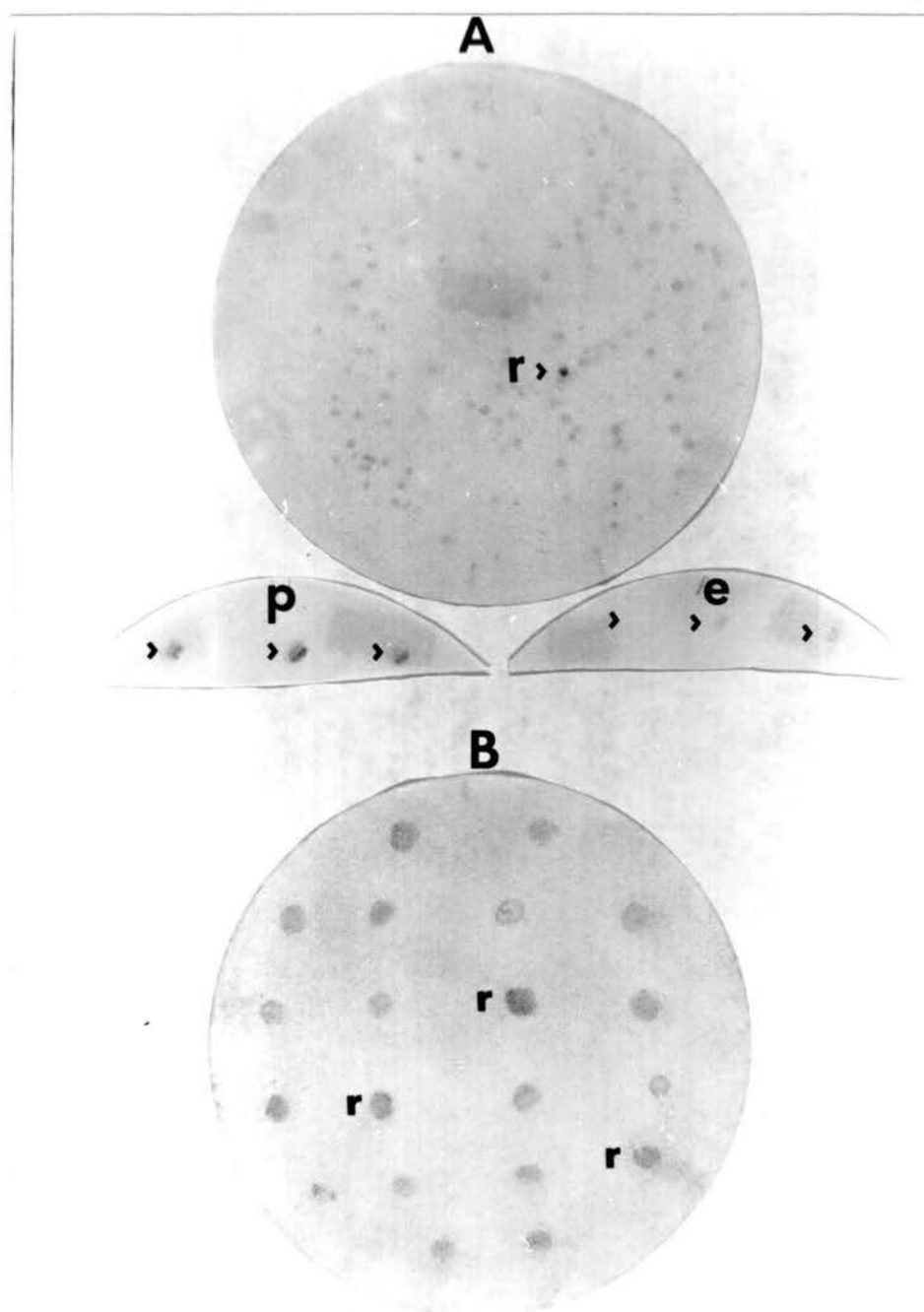


Figure 7. Gel and Blot for Southern Hybridization of EcoRI
Digested Clones
Lane a: HindIII-digested λ DNA size marker
Lane b: pWE15 digested with EcoRI
Lane c through g: EcoRI digested
recombinant clones hybridizing with UA2
Lane h: EcoRI digested pUC19 with insert

a b c d e f g h



a b c d e f g h

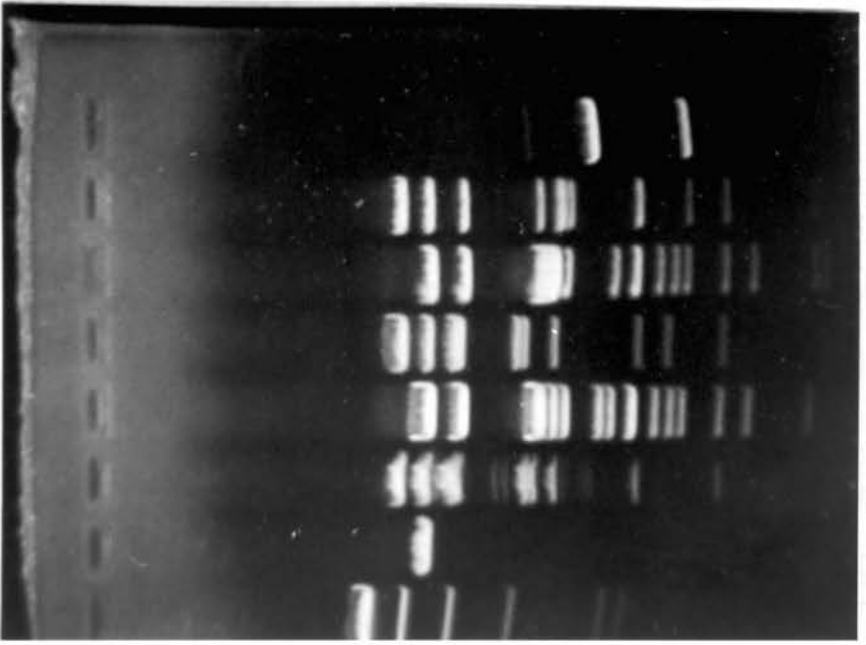
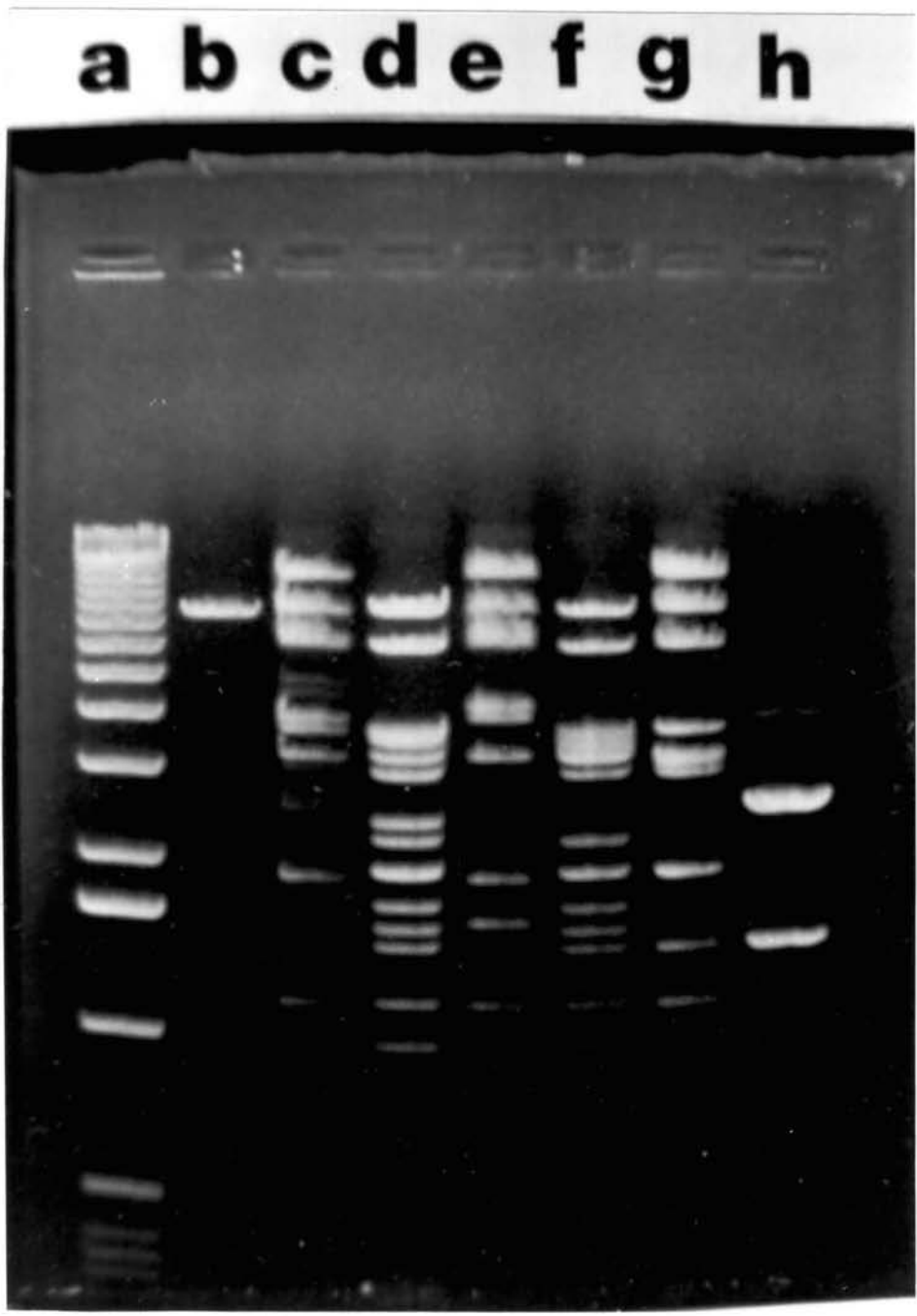


Figure 8. Agarose Gel Electrophoresis of EcoRI
Restriction Digestion of the 5 Positive
Clones
Lane a: 1 Kb DNA ladder
Lane b: EcoRI-digested pWE15 DNA
Lane c through g: EcoRI digests of
clones 1, 2, 3, 4 and 5 respectively
Lane h: EcoRI-digested pUC19 with
insert



Clone 3 showed a 9.5 Kb hybridizing EcoRI fragment. The clone has 10 EcoRI fragments and the insert size is 32.3 Kb.

Clone 4 showed a 3.4 Kb EcoRI fragment that showed hybridization with UA2. The clone has 17 EcoRI fragments and the insert size is 39.4 Kb.

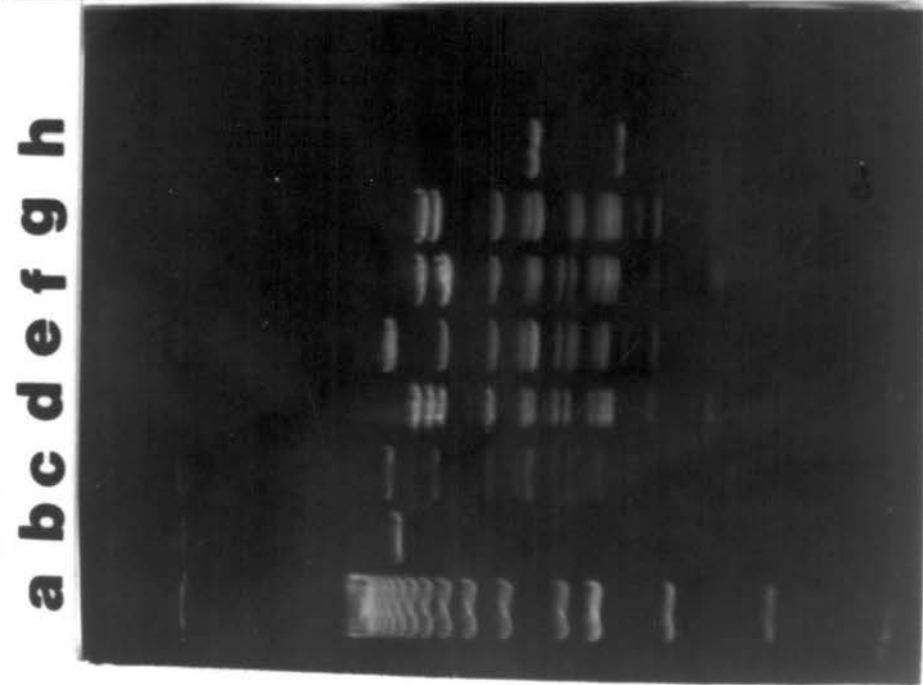
Clone 5 showed a 9.5 Kb EcoRI hybridizing fragment. The clone has 10 EcoRI fragments and the insert size is 30.5 Kb.

HindIII-digestion and Southern Hybridization: The 5 positive clones were digested with HindIII, blotted and hybridized with UA2. All clones showed hybridization of a 1.6 Kb HindIII fragment with UA2. The results of the hybridization are shown in Figure 9.

Digestion by Low-Frequency-Cutting Restriction Enzymes

In a previous study by Allardet-Servent et al. (2) it was shown that whole Brucella DNA cleaved with NotI gave nearly 40 bands on the restriction pattern i.e. NotI is a low-frequency-cutting restriction enzyme. This enzyme which has an 8-base-pair recognition site was incubated with DNA extracted from the 5 clones in a digestion reaction to determine if the inserts had any NotI sites. No restriction bands were obtained after overnight incubation.

Figure 9. Gel and Blot for Southern Hybridization of
HindIII-digest of 5 Clones
Lane a: 1 Kb DNA ladder
Lane b: pWE15 digested with EcoRI
Lane c through g: HindIII-digest of clones
1, 2, 3, 4 and 5 respectively
Lane h: EcoRI-digest of pUC19 with insert



XbaI which has a 6-base-pair recognition site that occurs relatively rarely in various DNAs, also did not cut within any of the 5 clones.

The same result was obtained with XhoI, another enzyme that has a 6-base-pair recognition site. Both XbaI and XhoI had been used to digest DNA from various Brucella strains and showed 25 to 30 restriction bands (2).

Digestion with BamHI

The 5 clones were digested with BamHI with the following results:

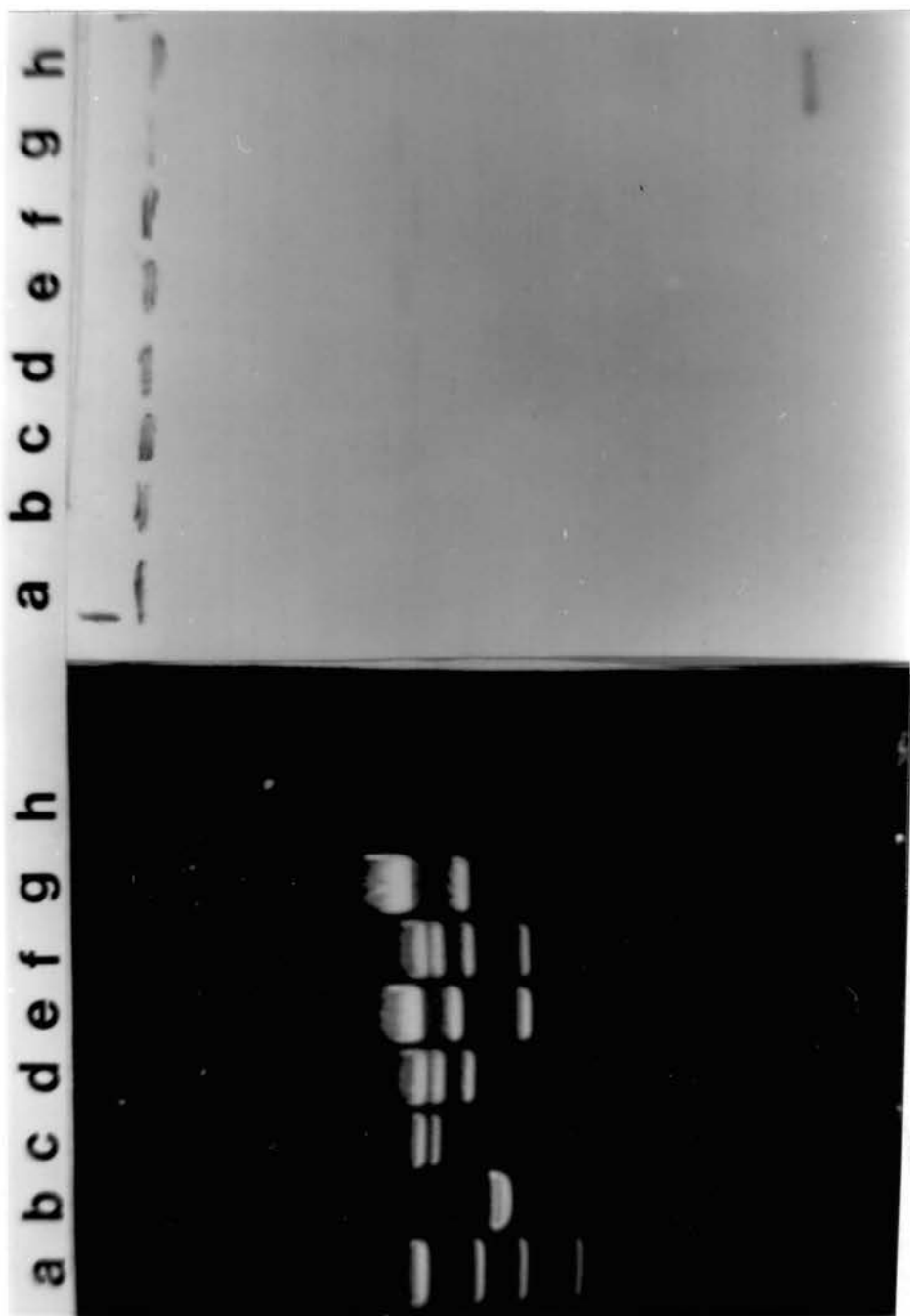
Clone 1 showed 2 restriction bands, clone 2 showed 3 bands, clone 3 showed 3 bands, clone 4 showed 4 bands and clone 5 showed 2 bands. No bands corresponding to pWE15 were seen and apparently the BamHI cloning sites were destroyed after ligation.

The BamHI digested clones were blotted and probed with UA2. The results showed homology of UA2 with a 23.8 Kb BamHI fragment in clone 1, a 16.5 Kb fragment in clone 2, a 22.2 Kb fragment in clone 3, a 16.5 Kb fragment in clone 4 and 28 Kb fragment in clone 5. The results of the hybridization are shown in Figure 10.

Hybridization with Labeled pWE15

A BamHI digest of the 5 positive clones was blotted and probed with biotinylated pWE15 to locate the fragments that hybridized with both pWE15 and UA2. pWE15 hybridized

Figure 10. Gel and Blot for Southern Hybridization of BamHI-Digested Clones Probed with UA2
Lane a: HindIII-digested lambda DNA size marker
Lane b: pWE15 digested with BamHI
Lane c through g: BamHI digests of clones 1, 2, 3, 4 and 5 respectively
Lane h: UA2 insert DNA



with a 15.4 Kb BamHI fragment in clone 1, a 16.5 Kb fragment in clone 2, a 12.6 Kb fragment in clone 3, a 16.5 Kb fragment in clone 4 and a 28 Kb fragment in clone 5. The results are shown in Figure 11.

Restriction Mapping of the Positive Clones

Upon comparison of the results of hybridization of the clones with pWE15 and with UA2 it was found that in each one of clones 2, 4 and 5 both pWE15 and UA2 hybridized to the same BamHI fragment. In clone 2 they both hybridized to a 16.5 Kb BamHI fragment, in clone 4 the hybridization was to a 16.5 Kb BamHI fragment and in clone 5 they both hybridized to a 28 Kb fragment. Comparison of the BamHI digests of the 5 clones also showed 3 BamHI fragments that were common in different clones which shows that these clones are overlapping. A 14.1 Kb fragment is common in clone 2 and 4, an 11.2 Kb fragment is common in clones 2, 4 and 5 and a 6.3 Kb fragment is common in clones 3 and 4. From the above mentioned information a restriction map for the positive clones is proposed as shown in Figure 12.

Screening with Monoclonal Antibody

The 5 colonies shown to be positive upon Southern hybridization were screened with a monoclonal antibody prepared against B. abortus O-antigen. All 5 colonies

Figure 11. Gel and Blot for Southern Hybridization of BamHI-Digested Clones Probed with pWE15
Lane a: HindIII-digested lambda DNA size marker
Lane b: pWE15 digested with BamHI
Lane c through g: BamHI digests of clones 1, 2, 3, 4 and 5 respectively
Lane h: EcoRI digested pUC19 with insert

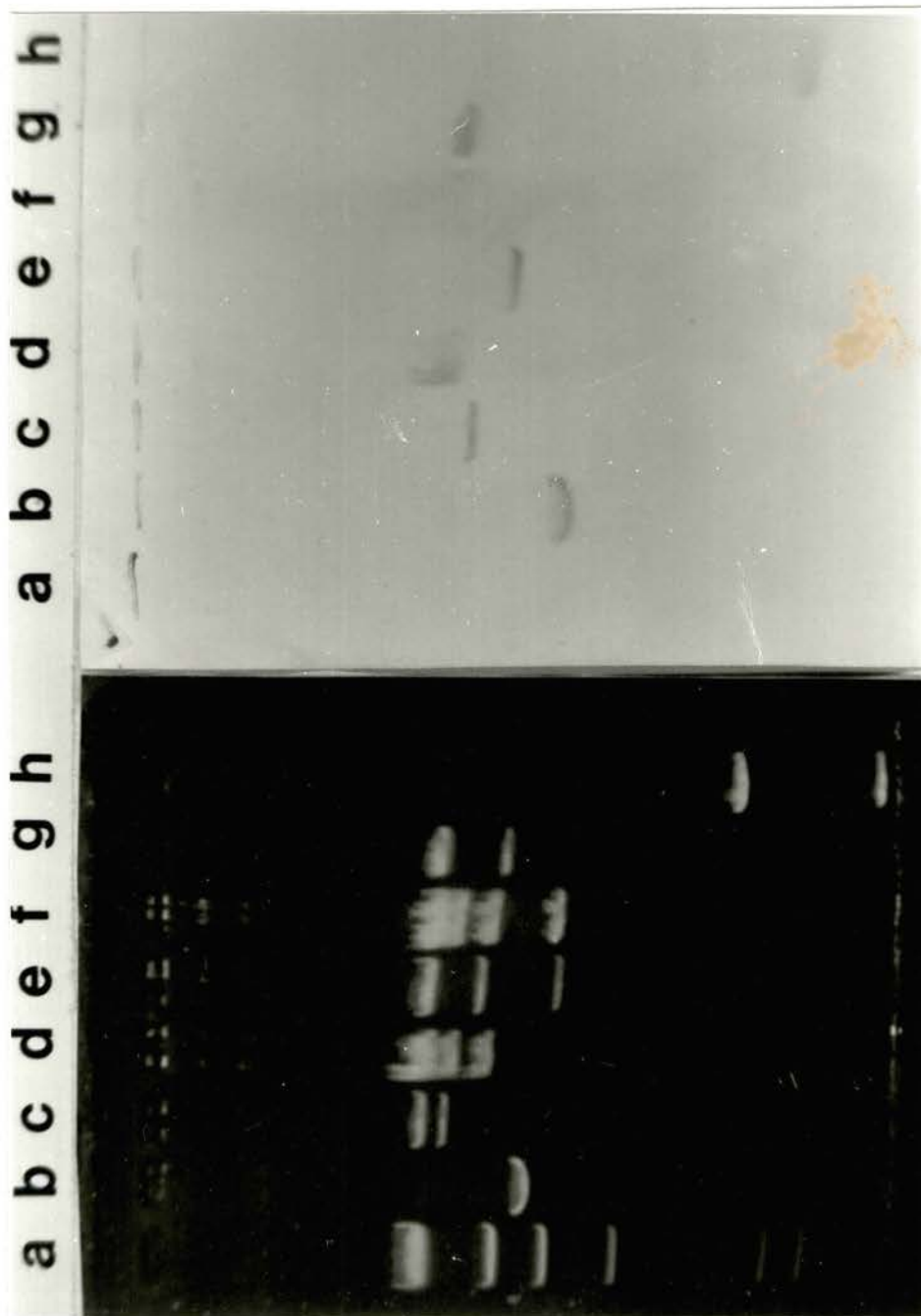
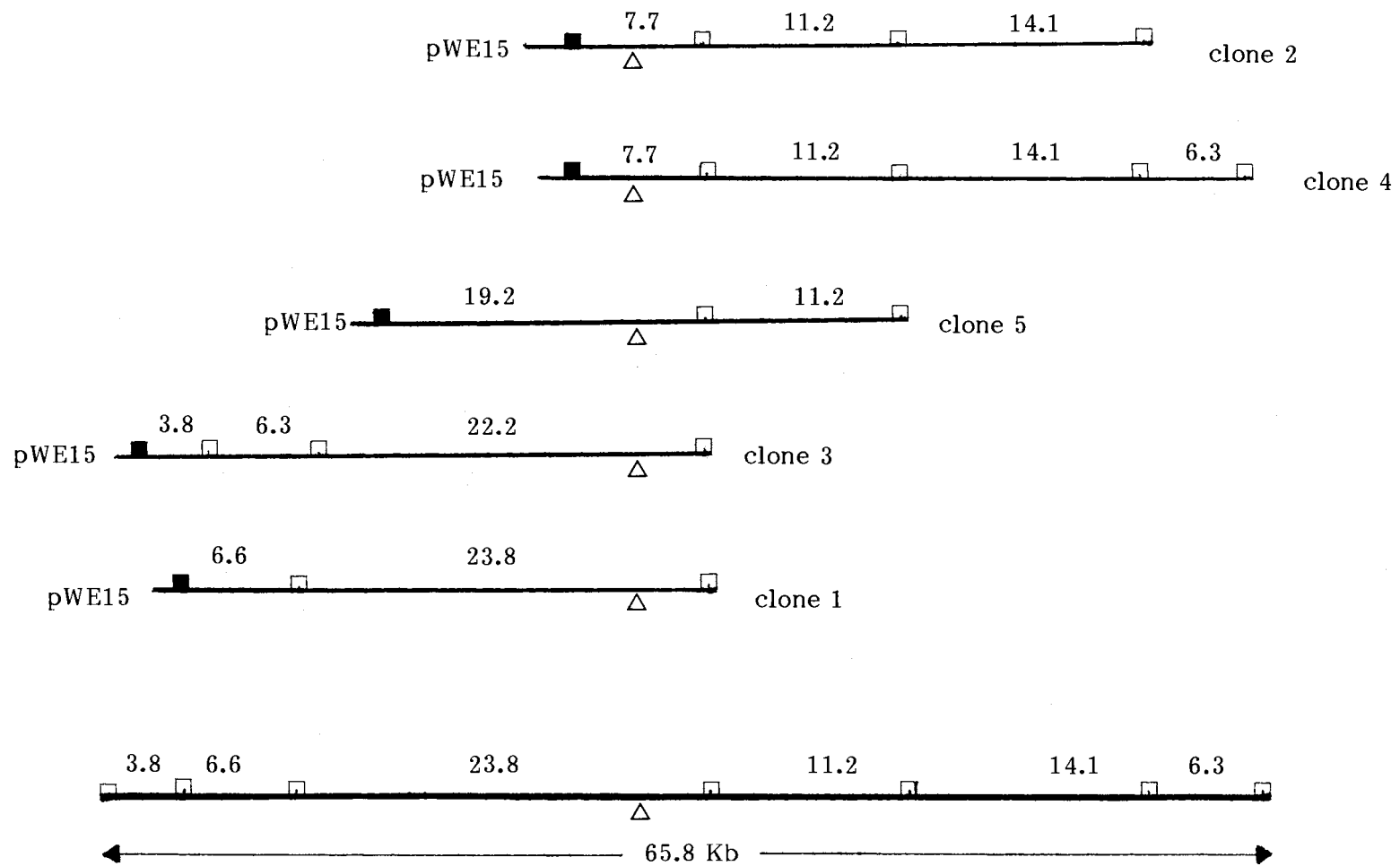


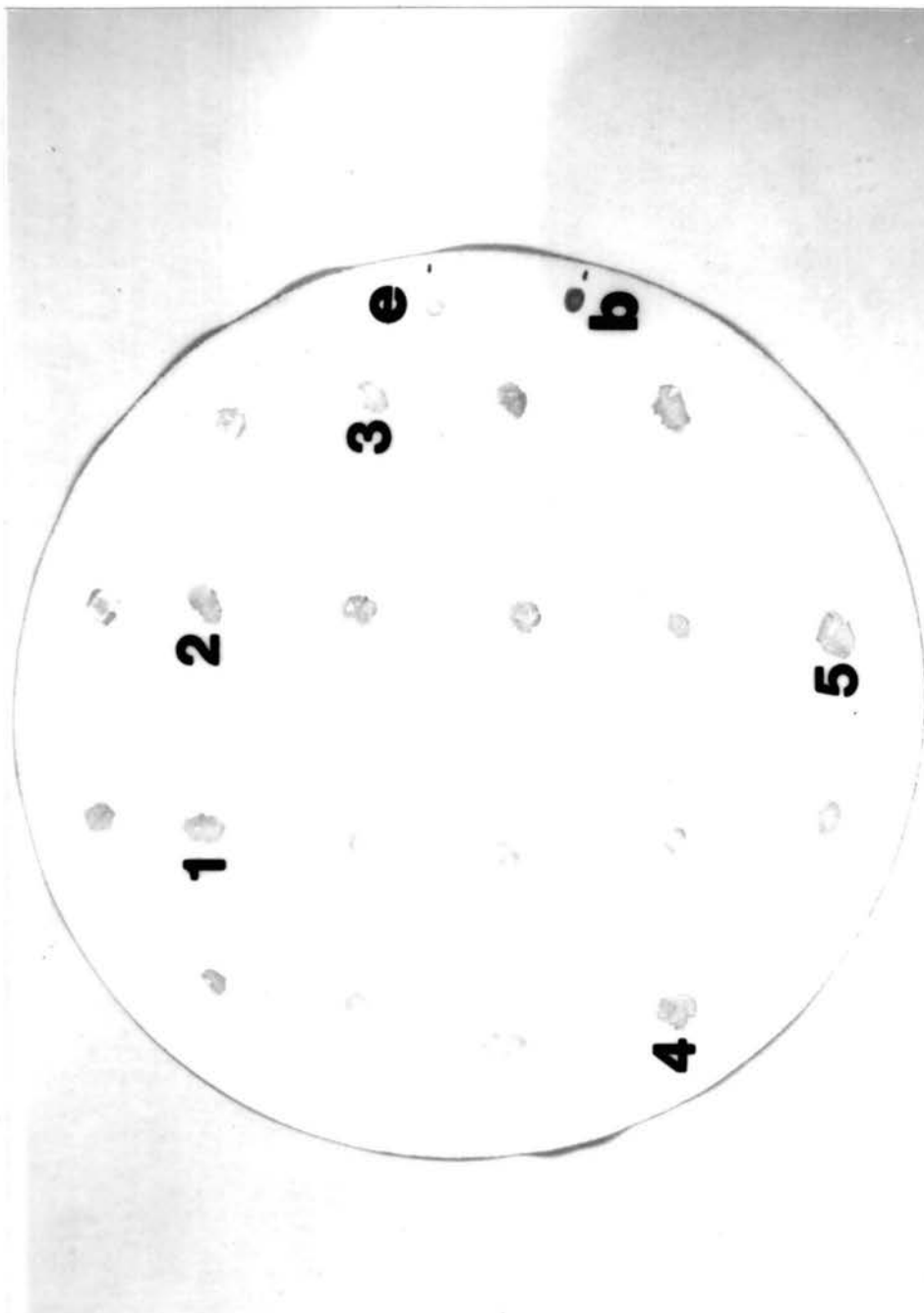
Figure 12. Proposed Restriction Map for the Positive Clones

- : Intact BamHI site
- : Destroyed BamHI site
- △: UA2



showed a positive reaction with the monoclonal antibody, as shown in Figure 13.

Figure 13. Immunoblot of 20 Colonies on a Nitrocellulose Filter Using Anti-Brucella O-antigen Monoclonal Antibody
e: E. coli cells as negative control
b: Brucella cells as positive control
1, 2, 3, 4, 5: Clones 1, 2, 3, 4, 5, respectively.



CHAPTER IV

DISCUSSION

The aim of this study was to construct a cosmid library of B. abortus genes in E. coli and to localize the genes coding for LPS production.

The analysis of complex genomes regarding their structure and function usually requires the isolation and physical mapping of large regions of DNA. Cosmids are the vectors of choice in this analysis, because they can accommodate genomic DNA fragments ranging in size from 30 to 45 Kb. Cloning large DNA segments has several advantages. Larger genes can be isolated on a single recombinant clone, several linked genes can be isolated on the same recombinant molecule, genes can be isolated with large stretches of surrounding sequences, and fewer colonies need to be screened to isolate the clone of interest. Another advantage is the procedure of genome 'walking' which allows the isolation of sequentially overlapping cosmid clones as well as the physical linkage and extensive characterization of genes (12).

Despite these advantages, cosmid cloning presents three main problems that have been overcome by the system used in this study as described by Dilella and Woo (12).

The first problem is vector to vector ligation resulting in cosmids lacking insert DNA. This was overcome by treating linearized cosmid DNA with alkaline phosphatase which prevents self ligation of vector DNA. Second, recombinational rearrangement of the insert DNA may take place due to ligation of multiple inserts into a single cosmid. This problem can be minimized by selecting insert fragments of a certain size (30-40 Kb) for ligation to the cosmid vector. Insertion of 2 or more such fragments into the same cosmid will result in a molecule too large to be packaged into a bacteriophage lambda particle. The third problem is differential growth of cosmid clones causing misrepresentation of insert sequences in amplified cosmid libraries. Related to this is the problem of unstable sequences or sequences that re-arrange spontaneously or become smaller. The cause of this can be any of several reasons; operator homologies of vector and insert, inverted duplication and repressor binding sites (35). Deletions can be avoided by preserving cosmids as early as possible after isolation and by scaling up cultures slowly if large scale DNA preparations are required.

Previous studies demonstrated the cloning of Brucella genes in E. coli. Mayfield et al. demonstrated the cloning and expression of a gene coding for a 31 Kilo Dalton immunogenic B. abortus protein. The protein was expressed well, apparently from its native promoter, when placed in different E. coli plasmids, i.e. Brucella promoters are

capable of functioning in E. coli. Sanborn et al. (personal communication) described the construction of a λ gt11 library of B. abortus S19 DNA in E. coli, indicating that Brucella genes can be cloned and expressed in E. coli. Clones were isolated which reacted to a monoclonal antibody against purified Brucella LPS. The specific epitope recognized was shown to resist pronase and proteinase K but was sensitive to treatment with periodate indicating that it was carbohydrate in nature and it was concluded that the LPS insert isolated, (UA2), was part of the O-antigen synthesis pathway. It was also shown that the UA2 insert DNA was found in all species of Brucella. The antigen was affinity-purified with a monoclonal antibody and used to immunize mice which were then challenged with live Brucella. The mice vaccinated with the recombinant antigen were protected against the Brucella challenge. Inserts in the λ gt11 library were about 0.5 to 3.0 Kb in size. These inserts are too small to represent the whole DNA sequence necessary for LPS synthesis.

Cloning of LPS has been reported in other Gram-negative organisms. Palermo et al. (51) demonstrated cloning and expression of the O-antigen from Neisseria gonorrhoeae by cloning a 9 Kb DNA fragment into the plasmid pBR322 and transformation of E. coli HB101. The antigen did not show reactivity to anti-outer membrane protein antibody, and showed resistance to proteinase K digestion, indicating that it was not protein. Electrophoresis of the

cloned gene products on urea-SDS-PAGE gels followed by Western blotting showed gel patterns and antibody reactivities similar to N. gonorrhoeae O-antigen.

Manning et al. (41) described the molecular cloning and expression in E. coli K-12 of the genes that determine the O-antigen of Vibrio cholerae. Genomic fragments of approximately 40 Kb were cloned into the cosmid pHc79 and E. coli were infected. Three clones were isolated from the library and were shown to express Vibrio cholerae O-antigen genes as determined by antibody reactions, PAGE and Western blot analysis. The results show that the enzymes involved in O-antigen biosynthesis can function in E. coli K-12 which has a defective rfb (O-antigen biosynthesis) region. It was reported that the synthesis of the O-antigen of Vibrio cholerae is determined by a gene cluster.

Kadam et al. demonstrated the cloning of rfa G, B, I, and J genes for glycosyltransferase enzymes for synthesis of the LPS core of Salmonella typhimurium. They proposed that a cluster of genes found in a 10 Kb fragment is responsible for the synthesis of the LPS core region (25).

Based on the results of cloning of LPS genes in N. gonorrhoeae, V. cholerae and S. typhimurium it was assumed in this study that the genes coding for LPS synthesis in Brucella would be found in a cluster. Thus, a cosmid vector was used to clone large DNA segments which would increase the probability of cloning the complete sequence coding for LPS production. The library constructed in this

study contains 30-40 Kb fragments of the B. abortus genome cloned into the cosmid pWE15. The host cells used, E. coli Ag1, are O-antigen deficient which allows assessment of cloning of the Brucella O-antigen. The library was calculated to contain 1.4×10^4 recombinant colonies. Based on the size of the Brucella genome, only 800 colonies are required to represent the whole Brucella genome which indicates that the library constructed here should contain any given Brucella DNA sequence. To detect expression of Brucella antigens the library was screened with rabbit polyclonal anti-whole Brucella antibody. More than 80% of the colonies screened showed expression of antigens which recognized the polyclonal antibody.

Hybridization of the recombinant colonies with the previously isolated UA2 insert showed that 20 colonies had DNA homology with UA2. Upon Southern blotting and hybridization, 5 clones showed homology with UA2.

Low-frequency-cutting enzymes, XhoI, XbaI and NotI were used to digest the clones but none of them showed any restriction bands. When BamHI was used to digest the clones a small number of digestion fragments was produced which would facilitate mapping of the clones. However, this was complicated by the fact that no BamHI fragments corresponding to pWE15 were recovered. In a study by Little (35), it was shown that fusion of Sau3A and BamHI site destroys the BamHI site in most cases, making it harder to analyse the extreme ends of the insert and to

identify cosmid overlaps. Since recognition sequences for the restriction enzyme NotI are present in the vector near the BamHI cloning site, and as NotI is a low frequency cutting enzyme, it can be used to remove the inserts as a single large fragment (12).

An alternative method for identification of overlapping clones is the use of bacteriophage T3 and T7 promoters flanking the BamHI cloning site. By using the cosmid DNA containing a genomic insert as a template for either T3 or T7 polymerase, labeled RNA probes that are specific for the extreme ends of the DNA can be synthesized. These probes can be used then for the identification of new clones that overlap the starting one. This process of 'walking' can reduce the requirement to identify ends of clones by restriction mapping (12).

Hybridization of BamHI digests of the 5 clones with labeled UA2 and with labeled pWE15 demonstrated that in three clones (clones 2, 4 and 5), both probes hybridized to the same band, a 16.5 Kb fragment in clone 2, a 16.5 Kb fragment in clone 4 and a 28 Kb fragment in clone 5. Three other BamHI fragments were common to more than one clone. Thus, a restriction map was proposed which shows that the 5 clones are probably overlapping, and that large DNA stretches, >25 Kb on one side of UA2 and >24 Kb on the other side have been cloned. The results of the previous studies on cloning of LPS genes in other Gram-negative bacteria, showed that in S. typhimurium, a 10 Kb fragment

is responsible for synthesis of LPS core region and in V. cholerae a 15 Kb DNA fragment determines the biosynthesis of 2 serotypes of the O-antigen. It can therefore be proposed that a 50 Kb stretch of DNA enclosing the UA2 sequence most likely contains the genes required for LPS synthesis assuming that they are located in a cluster.

The 5 clones were tested with anti-Brucella O-antigen monoclonal antibodies. All 5 clones showed a positive reaction which confirmed the results obtained by Southern hybridization.

Construction of B. abortus genomic library in E. coli will allow for the genetic study of Brucella organisms without the dangers and restrictions of direct handling of the organisms. It is easier and more economical to work with E. coli than with Brucella.

There are several advantages for cloning of genes coding for Brucella LPS synthesis. Cloning of LPS genes will allow the study of the structure and properties of LPS to be carried out more easily. Large amounts of purified LPS would be produced, for the study of LPS alone without the adherent Brucella protein so the function of each could be assessed separately. As mentioned before, the differences in structure and biological properties between E. coli and Brucella LPS will allow for the differentiation between E. coli LPS and the cloned Brucella LPS. Also the clones and purified Brucella LPS should be compared to the native Brucella LPS and characterized regarding its physi-

cal and chemical properties. Immunological characterization should also be performed through Western blots.

The recombinant LPS may be used as a component of a subunit vaccine against brucellosis which would avoid the side effects caused by injection of whole living organisms. Vaccination of mice could be performed with the recombinant product followed by challenge of the mice with Brucella organisms. These experiments would assess the role of cloned LPS in protection of mice. Further experiments should then be performed on cattle.

Also the recombinant LPS would be used to evaluate its role in the pathogenicity of Brucella and its survival within host macrophages.

In conclusion, a genomic library of B. abortus S19 genes has been constructed in the cosmid pWE15, the DNA packaged in vitro and transduced in E. coli. The recombinant fragments averaged 30-40 Kb in size and the titer of the library is sufficient to represent all sequences in the Brucella genome. More than 80% of the recombinant colonies express Brucella antigens as shown by screening with anti-whole Brucella antibody. Five clones were isolated which showed DNA homology with a 1.5 Kb Brucella LPS insert. The 5 clones produced overlapping BamHI fragments, making it possible to propose a restriction map for these clones. The 5 clones were tested with a monoclonal antibody specific for Brucella O-antigen and were shown to be positive. Assuming that the genes for LPS synthesis are

clustered, sufficient DNA on either side of the UA2 has been cloned to include the LPS gene cluster.

LITERATURE CITED

1. Abdelnoor, A.M. 1983. Effect of LPS on the clotting system. In: Beneficial effects of endotoxins. Plenum Press, New York.
2. Allerdet-Servent, A., G. Bourg, M. Ramuz, M. Pages, M. Bellis, and G. Roizes. 1988. DNA polymorphisms in strains of the genus Brucella. J. Bacteriol. 17: 4603-4607.
3. Baker, P.J., and J.B. Wilson. 1965. Chemical composition and biological properties of the endotoxin of Brucella abortus. J. Bacteriol. 90: 895-902.
4. Baker, P.J., and J.B. Wilson. 1965. Hypoferremia in mice and its application to the bioassay of endotoxin. J. Bacteriol. 90: 903-910.
5. Bates, P. 1987. Double cos site vectors: simplified cosmid cloning. In: Methods in enzymology. 153: 82-94.
6. Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513-1523.
7. Bradley, S.G. 1979. Cellular and molecular mechanisms of action of bacterial endotoxins. Ann. Rev. Microbiol. 33: 67-94.
8. Caroff, M., D.R. Bundle, M.B. Perry, J.W. Cherwonogrodzky, and J.R. Duncan. 1984. Antigenic S-type lipopolysaccharide of Brucella abortus 1119-3. Infect. Immun. 46: 384-388.
9. Christie, A.B. 1980. Brucellosis. Undulant fever. Malta or Mediterranean fever. In: Infectious diseases. Epidemiology and Clinical Practice. Third edition. Churchill Livingstone.
10. Davis, B.D., R. Dulbecco, H.N. Eisen, and H.S. Ginsberg. 1980. Microbiology. Third edition. Harper and Row, Publishers, Inc.

11. Diaz, R. L.M. Jones, D. Leong, and J.B. Wilson. 1968. Surface antigens of smooth Brucellae. J. Bacteriol. 96: 893-901.
12. Dilella, A.G., and S.L.C. Woo. 1987. Cloning large segments of genomic DNA using cosmid vectors. In: Methods in enzymology. 152: 199-212.
13. Duncan, R.L., Jr., J. Hoffman, V.L. Tesh, and D.C. Morrison. 1986. Immunologic activity of LPS released from macrophages after the uptake of intact E. coli in vitro. J. Immunol. 136:8-15.
14. Evans, G.A., and G.M. Wahl. 1987. Cosmid vectors for genomic walking and rapid restriction mapping. In: Methods in enzymology. 152: 604-610.
15. Ficht, T.A., S.W. Bearden, B.A. Sowa, and L.G. Adams. 1988. A 36-kilodalton Brucella abortus cell envelope protein is encoded by repeated sequences closely linked in the genomic DNA. Infect. Immun. 56: 2036-2046.
16. Forster, A.C., J.L. McInnes, D.C. Skingle and R.H. Symons. 1985. Non-radioactive hybridization probes prepared by the chemical labelling of DNA and RNA with a novel reagent, photobiotin. Nucleic Acid Res. 13: 745-761.
17. Freeman, A.B., D.J. Kross and R. Circo. 1961. Host parasite relationships in brucellosis. J. Inf. Dis. 108: 333-338.
18. Freeman, A.B., and L.R. Vana. 1958. Host-parasite relationships in Brucellosis. J. Inf. Dis. 102: 258-267.
19. Ganado, W. 1965. Human brucellosis: some clinical observations. Scott. Med. J. 10: 451-455.
20. Holland, J.J. and M.J. Pickett. 1956. Intracellular behavior of Brucella variants in chick embryo cells in tissue culture. Proc. Soc. Exper. Biol. and Med. 93: 476-479.
21. Hurvell, B. 1973. Serological cross-reactions between different Brucella species and Yersinia enterocolitica Acta path. microbiol. Scand. B, 81: 105-112.
22. Ish-Horowicz, D., and J.F. Burke. 1981. Rapid and efficient cosmid cloning. Nucleic Acids Res. 9: 2989-2998.

23. Jones, L.M., and D.T. Berman. 1976. Studies of Brucella lipopolysaccharide. International Symposium on Brucellosis (II). Dev. Biol. Stand. 31: 62-67.
24. Jones, L.M., R. Diaz, and D.T. Berman. 1976. Endotoxic activity of rough organisms of Brucella species. Infect. Immun. 6: 1638-1641.
25. Kadam, S.K., A. Rehemtulla, and K.E. Sanderson. 1985. Cloning of rfa G, I, and J Genes for Glycosyltransferase enzymes for synthesis of the lipopolysaccharide core of Salmonella typhimurium. J. Bacteriol. 161: 277-284.
26. Keleti, G., D.S. Feingold, and J.S. Youngner. 1974. Interferon induction in mice by lipopolysaccharide from Brucella abortus. Infect. Immun. 10: 282-283.
27. Keppie, J., A.E. Williams, K. Witt, H. Smith. 1965. The role of erythritol in the tissue localization of the Brucellae. Br. J. Exp. Path. 46: 104-108.
28. Keppie, J., K.A. Witt, H. Smith. 1966. Early appearance of colonies of Brucella species on solid media containing erythritol. J. appl. Bact. 29: 556-558.
29. Kreutzer, D.L., C.S. Buller, and D.C. Robertson. 1979. Chemical characterization and biological properties of lipopolysaccharides isolated from smooth and rough strains of Brucella abortus. Infect. Immun. 23: 811-818.
30. Kreutzer, D.L., and D.C. Robertson. 1979. Surface macromolecules and virulence in intracellular parasitism: comparison of cell envelope components of smooth and rough strains of Brucella abortus. Infect. Immun. 23: 819-828.
31. Kreutzer, D.L., L.A. Dreyfus, and D.C. Robertson. 1979. Interaction of polymorphonuclear leukocytes with smooth and rough strains of Brucella abortus. Infect. Immun. 23: 737-742.
32. Leary, J.J., D.J. Brigati and D.C. Ward. 1983. Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose bioblots. Proc. Nat. Acad. Sci. USA. 80: 4045-4049.

33. Leong, D., R. Diaz, K. Milner, J. Rudbach, and J.B. Wilson. 1970. Some structural and biological properties of Brucella endotoxin. *Infect. Immun.* 1: 174-182.
34. Limet, J., A.M. Plommet, G. Dubray, and M. Plommet. 1987. Immunity conferred upon mice by anti-LPS monoclonal antibodies in marine brucellosis. *Ann. Inst. Pasteur. Immunol.* 138: 417-424.
35. Little, P.F.R. 1988. Choice and use of cosmid vectors. In: *Vectors: A survey of molecular cloning vectors and their uses.* (R. Rodriguez, ed.). Butterworth, London.
36. Lopez-Merino, A., J. Asselineau, A. Serre, J. Roux, S. Bascoul, and C. Lacave. 1976. Immunization by an insoluble fraction extracted from Brucella melitensis. Immunological and chemical characterization of the active substances. *Infect. Immun.* 13: 311-321.
37. Luderitz, O., C. Galanos, V. Lehman. 1973. Lipid A, chemical structure and biological activity. *J. Inf. Dis.* 128: (suppl. 17) 17-29.
38. Luderitz, O., O. Westphal, A.M. Staub, and H. Nikaido. 1971. Isolation and chemical and immunological characterization of bacterial lipopolysaccharides. In: *Microbial toxins, vol. IVb*, New York, Academic Press.
39. Mandell, G.L., R.G. Douglas, Jr., J.E. Bennet. 1985. *Principles and practice of Infectious diseases.* Wiley medical publication. John Wiley and Sons.
40. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. *Molecular cloning, a laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
41. Manning, P.A., M.W. Heuzenroeder, J. Yeadon, D.I. Leavesley, P.R. Reeves, and D. Rowley. 1986. Molecular cloning and expression in Escherichia coli K-12 of the O antigens of the Inaba and Ogawa serotypes of the Vibrio cholerae O1 lipopolysaccharide and their potential for vaccine development. *Infect. Immun.* 53: 272-277.

42. Mayfield, J.E., B.J. Bricker, H. Godfrey, R.M. Crosby, D.J. Knight, S.M. Halling, D. Balinstey, and L.B. Tabatabai. 1988. The cloning, expression, and nucleotide sequence of a gene coding for an immunogenic Brucella abortus protein. *Gene*. 63: 1-9.
43. McCullough, N.B. 1970. Microbial and host factors in the pathogenesis of brucellosis. In: Mudd S. (ed) *Infectious agents and host reactions*. W.B. Saunders, Philadelphia.
44. Möller, G., O. Sjöberg, and J. Anderson. 1973. Immunogenicity, tolerogenicity and mitogenicity of lipopolysaccharides. p. 44-48. In: *Bacterial lipopolysaccharides*. The University of Chicago Press, Chicago, Ill.
45. Montaraz, J.A., A.J. Winter, D.M. Hunter, B.A. Sowa, A.M. Wu, and L.G. Adams. 1986. Protection against Brucella abortus in mice with O-polysaccharide-specific monoclonal antibodies. *Infect. Immun.* 51: 961-963.
46. Moreno, E., and D.T. Berman. 1979. Brucella abortus lipopolysaccharide is mitogenic for spleen cells of endotoxin-resistant C3H/HeJ mice. *J. Immunol.* 123: 2915-2919.
47. Moreno, E., D.T. Berman, and L.A. Boettcher. 1981. Biological Activities of Brucella abortus lipopolysaccharides. *Infect. Immun.* 31: 362-370.
48. Moreno, E., M.W. Pitt, L.M. Jones, G.S. Schurig, and D.T. Berman. 1979. Purification and characterization of smooth and rough lipopolysaccharides from Brucella abortus. *J. Bacteriol.* 138: 361-369.
49. Moreno, E., R.S. Kurtz, and D.T. Berman. 1984. Induction of immune and adjuvant immunoglobulin G responses in mice by Brucella lipopolysaccharide. *Infect. Immun.* 46: 74-80.
50. Munoz, J.J., R.K. Bergman, and K.E. Robbins. 1978. Comparison of the histamine hypersensitivity and the Limulus amoebocyte lysate tests for endotoxin activity. *Infect. Immun.* 22: 292-294.
51. Palermo, D.A., T.M. Evans, and V.L. Clark. 1987. Expression of a cloned lipopolysaccharide antigen from Neisseria gonorrhoeae on the surface of E. coli K12. *Infect. Immun.* 55: 2844-2849.

52. Raff, R.A. and R.W. Wheat. 1968. Carbohydrate composition of the phenol soluble lipopolysaccharides of Citrobacter freundii. J. Bact. 95: 2035-2043.
53. Redfearn, M.S. 1960. An immunochemical study of antigens of Brucella extracted by the Westphal technique. Ph.D. thesis, University of Wisconsin, Madison, Wisconsin.
54. Riley, L.K. and D.C. Robertson. 1984. Brucellacidal activity of human and bovine polymorphonuclear leucocyte granule extracts against smooth and rough strains of Brucella abortus. Infect. Immun. 46: 231-236.
55. Riley, L.K. and D.C. Robertson. 1984. Ingestion and intracellular survival of Brucella abortus in human and bovine polymorphonuclear leukocytes. Infect. Immun. 46: 224-230.
56. Smith, H., and R.B. Fitzgeorge. 1963. The chemical basis of the virulence of Brucella abortus. Br. J. Exp. Path. 45: 174-186.
57. Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517.
58. Southern, P.J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. 1: 327-341.
59. Spellman, J.M. and N.D. Reed. 1979. Immune and mitogenic responses by Balb/c, C3H/HeJ, and nude mice to Brucella abortus bacteria and lipopolysaccharide. Infect. Immun. 24: 371-378.
60. Stinebring, W.R. and R. Kessel. 1959. Continuous growth of Brucella abortus in mononuclear phagocytes of rats and guinea pigs. Proc. Soc. Exp. Biol. Med. 101: 412-415.
61. Taub, F., and E.B. Thompson. 1982. An improved method for preparing large arrays of bacterial colonies containing plasmids for hybridization: in situ purification and stable binding of DNA on paper filters. Anal. Biochem. 126: 222-230.
62. Topley, W.W.C., and G.S. Wilson. 1964. Principles of bacteriology and immunity. Arnold, London.

63. Volk, W.A., D.C. Benjamin, R.J. Kadner, and J.T. Parsons. 1986. In: Essentials of medical microbiology. 3rd edition. J.B. Lippincot Company, Philadelphia.
64. Westphal, O., O. Luderitz, and F. Bister. 1952. Uber die Extraktion von Bakterien mit Phenol-Wasser. Z. Naturforsch. 7b, 148-155.
65. Winter, A.J., G.E. Rowe, J.R. Duncan, M.J. Eis, J. Widom, B. Ganem, and B. Morein. 1988. Effectiveness of natural and synthetic complexes of porin and O polysaccharide as vaccines against Brucella abortus in mice. Infect. Immun. 56: 2808-2817.
66. World Health Organization. 1964. Joint FAO/WHO Expert committee on Brucellosis. 4th Report. Tech. Rep. Serv. Wld. Hlth. Org. No. 289.

VITA

Mary Shokralla Sedra

Candidate for the Degree of

Master of Science

Thesis: MOLECULAR CLONING OF BRUCELLA ABORTUS GENES IN A COSMID VECTOR AND THEIR EXPRESSION IN ESCHERICHIA COLI

Major Field: Microbiology

Biographical:

Personal Data: Born in Alexandria, Egypt, August 25, 1952, the daughter of Shokralla N. Sedra and Aida I. Boulos. Married to Nasrat H. Soliman on April 12, 1977.

Education: Graduated from Lauranne High School, Alexandria, Egypt in June 1970; received Bachelor of Medicine and Surgery Degree from Alexandria University, Egypt in November 1976; completed requirements for the Master of Science Degree at Oklahoma State University in December 1989.

Professional Experience: House Officer in Alexandria University Hospitals, 1977-1978; General Practitioner in Ministry of Health Hospitals, Alexandria, 1978-1981; Resident in Clinical Pathology Laboratories, Alexandria, 1981-1984; Graduate Teaching Assistant, Department of Botany and Microbiology, Oklahoma State University, August 1986-July 1988; Graduate Research Assistant, Department of Botany and Microbiology, Oklahoma State University, August 1988-July 1989.